Interactions between above and below ground symbionts: Implications for food security

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PhD

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Biology

September 2018

Abstract

The below ground root symbiont arbuscular mycorrhizal fungi (AMF) can be involved in multitrophic interactions with plant associated pests, such as aphids. AMF can both increase and decrease aphid performance, but the underlying mechanisms are unclear. Mechanisms could include AMF altering 1) plant defence responses and signalling and/ or 2) the quality of the plant as a food source. Moreover, little is known about how aphid associated microbial facultative symbionts (FS) influence the outcome of the interaction, or how aphid herbivory affects the AMF colonising the plant.

To investigate the role of plant quality as a food source, nitrogen (N), a limiting nutrient in the aphid diet, was tracked through an AMF, barley (*Hordeum vulgare*) and English grain aphid (*Sitobion avenae*) glasshouse system. AMF increased the N concentration of the aphid's food source and it was demonstrated that AMF can deliver N to above ground organisms via the plant. However, aphid performance and N concentration remained unchanged. Metagenomic methods were used to investigate the impact of *S. avenae* on AMF colonising barley. In an agricultural system, the abundance of the AMF family Gigasporaceae tended to increase when aphids were present, suggesting that aphid herbivory could influence plant associated AMF communities.

To investigate the role of plant defence signalling, plant defence gene expression analysis was carried out in a broad bean (*Vicia faba*)-pea aphid (*Acyrthosiphon pisum*) system. AMF augmented the expression of a salicylic acid (SA) pathway gene (PR5) and reduced aphid performance. The FS *Hamiltonella defensa* did not alter the impact of AMF on the aphid.

This thesis contributes to the identification of driving mechanisms in AMF-aphid interactions and provides evidence that plant defence signalling can play a role. Moreover, in certain cases, aphids may be able to overcome changes in the N concentration of plant hosts caused by AMF.

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Acknowledgements

I would first like to thank my supervisors Julia Ferrari, Angela Hodge and Sue Hartley for their advice, support and direction throughout this journey. Not only have I been able to rely on your invaluable scientific expertise and experience, but I would also like to thank you for your feedback on writing this Thesis. With your generously given time, you have helped me improve, and I am sure I will take these skills into the future. Thank you all for giving me this opportunity. I would also like to thank the other academics who have supported me over the years. To my thesis advisory panel Calvin Dytham and Simon McQueen-Mason, your support and outside perspective during the progress of this project has kept me focused and encouraged. Thanks must also go to the other academics who have shared their time and expertise with me over the years such as Thorunn Helgason, Kelly Redeker, Michael Schultze, James Stockdale and Phil Ineson.

I have been lucky to work across multiple lab groups, and so work alongside many generous people who have extended their advice and friendship. I would like to thank those who worked with aphids; Mel, Ellie, Sally, Hannah and Megan - who helped me get to grips with the little creatures! I would like to thank the D0, J1 and M2 lab groups past and present; Erin, Emma, Pierre, Sarah, Ruth, Kayla, Tom I, Adam, Tim, Leda, Joe, Sue B, Michaela, JT and more who have also always been pillars of support. A special mention must go to Tom T, Phil B, Pasky and Tom H. Never in a thousand years would I have been brave enough on my own to attempt the statistical and bioinformatic analysis that you helped me with. I would like to thank Nathan Innard, my long-term competitor and fellow duck feeder. From the start of our undergraduate degrees, our competition always pushed me to achieve and I even enjoyed helping you cart your belongings all over York throughout my PhD. I am also grateful to the many project students that have come and gone over the years, your enthusiasm has been contagious. I would also like to acknowledge the stores team at the University of York, for your patience and advice for my lists of chemical reagents and end of the day trips to restock them. Finally, if my plants had not grown then none of this would have been possible, so thankyou to the horticultural team.

I was also lucky to work at ADAS High Mowthorpe, where as soon as I arrived I felt part of the team. I will never forget the people I worked with, how welcomed I was, and the immensely informative and enjoyable experience. Thank you to Kate Storer for organising my placement there, and I’m sorry I kept on crashing the trucks.

I’d like to thank all my family, including my parents, grandparents, siblings and nephew and nieces. You have always given me support and I am grateful for the care packages, encouragement and days off from working. You have always been understanding about the commitments this project has required, so thank you. In particular I would like to thank

one of the strongest people I know, my Mother. Since ‘Horrible Science’ you have always nurtured my interest in the natural world, and even through difficult parts in life you have encouraged me to succeed. Finally, I would like to thank the other strongest person in my life, Lauren. Again, through tough times you have always encouraged me to finish this project. You have selflessly supported me, put up with late night and weekend working, listened to me talk science, chauffeured me around and most amazingly made the last two years of this project enjoyable. Thank you.

Author's Declaration

I, Thomas D J Wilkinson, declare that this thesis is a presentation of original work and I am the sole author. This work has not been presented for an award at this, or any other, University. All sources are acknowledged as references.

Chapter 2

The set up and harvest of the first experiment in Chapter 2 was carried out by James Wilson, a student initially in receipt of this PhD projects funding until August 2014. This study was a pilot experiment to test the root length colonisation by arbuscular mycorrhizal fungi of traditional and modern barley varieties. The samples from the harvest were stored by James Wilson, and the subsequent analysis of the samples was carried out by me (Thomas Wilkinson). The statistical analysis of the data from these samples was carried out by me.

Chapter 3

Chapter 3 was published by Functional Ecology as a research article in January 2019;

'Wilkinson, T.D.J., Ferrari, J., Hartley, S.E., and Hodge, A. (2019). Aphids can acquire the nitrogen delivered to plants by arbuscular mycorrhizal fungi. *Functional Ecology*. doi:10.1111/1365-2435.13283'.

Notes: This research article has been modified into a chapter format for this thesis and so contains figure, text, table, unit and layout formatting differences, minor spelling and grammatical differences, as well as altered reference styles and altered figure/ table/heading numbers compared to the final published manuscript. The supplementary information is presented in the thesis appendix, and the reference list is combined into a list with the other references found in this thesis. Acknowledgements, conflicts of interest, authors contribution and data accessibility statements found in the final published manuscript are not included in this thesis.

Thomas Wilkinson (me), Sue Hartley, Julia Ferrari and Angela Hodge contributed to the writing of the manuscript. Thomas Wilkinson (me) conducted the experiment and analysed the data.

1 General introduction

1.1 Rationale

As plants exist simultaneously in soil and above it, they interact with both above and below ground organisms (van Dam & Heil 2011). Via their effects on the plant, these above and below ground organisms can interact without coming into direct contact, altering the growth and development (Vestergard, Bjornlund & Christensen 2004; Gallou *et al.* 2011; Babikova *et al.* 2014a) and community structures (Gehring & Bennett 2009; Barto & Rillig 2010; Kong *et al.* 2016) of each other. These effects can cascade through further trophic levels (Bennett *et al.* 2016), and so the plant becomes a conduit for a network of multitrophic interactions between above and below ground organisms. As these organisms include plant pests (Yang *et al.* 2014; Mauch-Mani *et al.* 2017; Hoysted *et al.* 2018) or mutualists (Guerrieri *et al.* 2004; Ba *et al.* 2012; Ballhorn, Younginger & Kautz 2014; Barber & Gorden 2015), above and below ground interactions could have wide implications for plant productivity.

Faced with an increasing world population and more variable and unpredictable growing conditions due to global climate change, there is an urgent need to improve crop productivity and resilience to extreme weather. However, this needs to be achieved alongside a reduction in the negative environmental implications of intensive agriculture (FAO 2011; DEFRA 2018). For example, improper use of agro-chemical inputs such as fertilisers and pesticides can lead to environmental pollution (Pretty *et al.* 2005; Park *et al.* 2012), and detrimental effects to non-target organisms (Antwi & Reddy 2015; van der Sluijs *et al.* 2015), respectively. Through the beneficial effects of, for example, symbiotic below-ground microorganisms, on plant growth as well as the potential adverse impacts of these organisms on foliar pests, it is proposed that multitrophic interactions between above and below ground organisms could contribute to reducing the reliance of crop production on agro-chemical inputs (Pineda, Kaplan & Bezemer 2017; Thirkell *et al.* 2017).

Arbuscular mycorrhizal fungi (AMF; Glomeromycotina) (Spatafora *et al.* 2016), are obligate biotrophs which form symbiotic associations with around two thirds of vascular plants including the majority of non-brassicaceous crops (Hughes *et al.* 2008; Smith & Read 2008). AMF derive their fixed carbon (C) source from plant host photoassimilates, and are reliant on the plant host to complete their life cycle (Smith & Read 2008). In return, AMF may aid the plant in acquiring primary macronutrients such as nitrogen (N) (Johansen, Jakobsen & Jensen 1992), phosphorus (P) (Jakobsen, Abbott & Robson 1992; Harrison, Dewbre & Liu 2002) and potassium (Garcia & Zimmermann 2014; Garcia *et al.* 2017), as well as other important nutrients in healthy crop development (Liu *et al.*

2000; Wang *et al.* 2017). Alongside a nutritional role, AMF may alter the plants tolerance to abiotic stresses such as drought (Qiao *et al.* 2012), salinity (Aroca *et al.* 2013; Estrada *et al.* 2013) and heavy metal toxicity (Zhang *et al.* 2005). Moreover, via their impacts on shared host plants, AMF can influence plant resistance to microbial (Gallou *et al.* 2011; Nair *et al.* 2015) and arthropod (Song *et al.* 2013) pests.

Generally, AMF reduce the performance of plant pests sharing the same host plant (Koricheva, Gange & Jones 2009; Yang *et al.* 2014), termed mycorrhizal induced resistance (MIR). However, the reported 'bottom up' multitrophic effects of AMF upon aphids (Hemiptera: Aphidoidea) sharing the host plant are less clear (Table 1). Aphids are phloem feeding insects comprising over 4000 species. Around 100 of these species, some generalist and some specialised to host plants, cause significant damage to crops (Blackman & Eastop 2000), and aphids are thus important economic pests (Larsson 2005; Ramsden *et al.* 2017; Zapata *et al.* 2018). Aphids reduce crop yields (Yencho, Getzin & Long 1986; Tatchell 1989; Mirik *et al.* 2009) by: directly feeding upon phloem sap (Douglas 2006); excreting the excess carbohydrates from this diet as honeydew, which leads to the growth of sooty moulds on the plant (Chomnunti *et al.* 2014); vectoring plant viruses (Ng & Perry 2004); and altering plant resource allocation (Girousse *et al.* 2005).

Conventionally, aphids are controlled with chemical pesticides and the breeding of aphid resistant traits in crops identified as, or hypothesised to be based upon, resistance (R) gene interactions. However, aphid resistance to both conventional chemical control (Bass *et al.* 2014; Foster *et al.* 2014; Malloch *et al.* 2014), and R gene and R gene like plant resistance (Goggin, Williamson & Ullman 2001; Alt & Ryan-Mahmutagic 2013) have developed in certain populations. Furthermore, such intrinsic resistance may not be available in all crops (Jaouannet *et al.* 2014). Combined with concerns for the impact of pesticides on non-target organisms (Antwi & Reddy 2015; van der Sluijs *et al.* 2015), this highlights the need for alternative control mechanisms.

The variation in the outcome of AMF-aphid interactions (Table 1) limits the potential for harnessing AMF to supress aphids in agricultural systems. This variation could be due to different environmental and genetic contexts across studies, but developmental factors within each study system can also play a role. For example, plant and aphid age (Tomczak & Muller 2017; Tomczak & Muller 2018), whether the aphid age as a colony is mixed or the same (Maurya *et al.* 2018), the level of AMF establishment in the plant host root (Tomczak & Muller 2017; Maurya *et al.* 2018), and the spore density of AMF in the growth substrate (Meir & Hunter 2018b), can all influence the impact of AMF upon aphids.

If the variation in the outcome of AMF-aphid interactions is to be better understood, and the benefits of these interactions for agriculture to be realised, it is important to identify the

underlying physiological and chemical mechanisms that drive AMF-aphid interactions. Broadly, these potential driving mechanisms can be broken down into: 1) effects via the nutritional quality of plants as an aphid food source; and 2) effects via plant defences and defence signalling (van Dam & Heil 2011; Babikova *et al.* 2014a). Increased complexity arises when the impact of aphids upon AMF via the shared plant host is considered, alongside the ability of aphids to form their own microbial symbioses. Thus, Key Knowledge Gaps in our understanding of how AMF and aphids may interact via plant nutrition and defence, and how these interactions are influenced by increased complexity of the multitrophic system will be discussed in the remainder of this introduction (Figure 1).

Table 1. Reported outcomes of multitrophic interactions between AMF and aphids. NA = not measured in study, ↑ = increase, ↓ = decrease, → = no effect.

Investigated effects of multitrophic interaction	Outcome[3]	AMF colonisation effects on plant N and P concentration		AMF colonisation effects on plant defence	Notes/ reference
		N	P		
Direct effect of AMF on aphid performance[1] on the shared host plants	Increase	↓	NA	↑ glycosides	Field fungicide treatment altered plant amino acid profile, reduced AMF colonisation, aphid weight and embryo content; Gange and West (1994)
		NA	NA	NA	AMF colonisation increased aphid weight and fecundity under low and medium P fertilisation regimes; Gange, Bower and Brown (1999)
		→	→	Altered plant volatile profiles	AMF colonisation of plants previous to aphid infestation increased aphid performance; Babikova *et al.* (2014a)
		NA	NA	NA	Reduced aphid development and reproduction time on +AM plants across multiple varieties. Increased vascular bundle size and phloem feeding behaviour on +AM plants; Simon *et al.* (2017)
		↑	→	NA	Aphid relative growth rate and nymph body mass influenced by AMF colonisation and plant age; Tomczak and Muller (2017)
		NA	NA	Altered gene expression unlinked to altered aphid performance	Total aphid dry weight increased by high levels of AM colonisation; Maurya *et al.* (2018)
		→	Plant spp. dependent	↑ cardenolide sequestration by aphids over all treatments	Across plant spp., aphids had highest performance on plants inoculated with highest levels of AMF spores; Meir and Hunter (2018b)
	No effect	↑	↑	Altered plant volatile profiles	Babikova *et al.* (2014b)

		→	NA	NA	Grabmaier *et al.* (2014)
		↑	↑	NA	Williams, Birkhofer and Hedlund (2014)
		NA	NA	NA	Bennett *et al.* (2016)
		NA	NA	NA	Karley, Emslie-Smith and Bennett (2017)
	Decrease	NA	NA	NA	Guerrieri *et al.* (2004)
		↓	↑	→ caltapol	Wurst *et al.* (2004)
		→	→	NA	Hempel *et al.* (2009)
		→	→	Altered plant volatile profiles	AMF colonisation of plants post aphid infestation decreased aphid performance; Babikova *et al.* (2014a)
		↑	→	NA	Tomczak and Muller (2017)
		NA	NA	Altered gene expression unlinked to altered aphid performance	Reduced aphid dry weight at low levels of AMF colonisation when aphids in colony are the same age; Maurya *et al.* (2018)
		→	Plant spp. dependent	↑ cardenolide sequestration by aphids over all treatments	Across plant spp. aphids had highest performance on plants inoculated with highest levels of AMF spores; Meir and Hunter (2018b)
		NA	NA	NA	Aphid relative growth rate and nymph body mass influenced by AMF colonisation and plant age; Tomczak and Muller (2018)
Effects of AMF on aphid attraction to / aphid colonisation[2] of host plants	Increase	NA	NA	NA	Increased ingress and proliferation of aphids on +AM plants; Abdelkarim *et al.* (2011)
		NA	↑	NA	Increased ingress and proliferation of aphids on +AM plants; Ueda *et al.* (2013)
		→	→	Altered plant volatile profiles	Increased aphid attraction to +AM plant headspace; Babikova *et al.* (2014a)

		↑	↑	Altered plant volatile profiles	Increased aphid attraction to +AM plant headspace independent of P regime; Babikova *et al.* (2014b)
		NA	NA	NA	Increased aphid numbers on +AM field treatment; Colella *et al.* (2014)
		NA	NA	↑ polyphenol oxidase and peroxidase activity in glasshouse	In field, aphid colonisation of plants depended on AM colonisation and plant variety; Balog *et al.* (2017)
		NA	NA	NA	Increases in aphid growth rate and settlement depending on plant variety; Simon *et al.* (2017)
	No effect	↑	↑	NA	No effect on aphid ingress or colonisation; Wurst and Forstreuter (2010)
	Decrease	NA	NA	Altered plant volatile profiles	Aphids repelled by volatiles from plants connected to aphid infested donor plants by AM hyphal networks; Babikova *et al.* (2013a)
		NA	NA	Altered plant volatile profiles	Aphids repelled by volatiles from plants connected to aphid infested donor plants by AM hyphal networks within 24 hours; Babikova *et al.* (2013b)
		NA	NA	↑ polyphenol oxidase and peroxidase activity in glasshouse	In glasshouse aphid colonisation reduced on +AM plants; Balog *et al.* (2017)
Effects of AMF on aphid antagonist attraction to host plants and aphid antagonist	Increase	NA	NA	NA	Parasitoid wasps more attracted to +AM plants, even in absence of aphids; Guerrieri *et al.* (2004)
		→	→	NA	Parasitoid weight and development speed increased in +AM systems; Hempel *et al.* (2009)
		NA	NA	NA	Parasitoid wasps attracted by volatiles from plants connected to aphid infested donor plants by AM hyphal networks; Babikova *et al.* (2013a)

performance (indirect defences)		NA	NA	NA	Parasitoid attack and emergence highest on aphids reared on AMF plants, in certain plant genotypes; Bennett *et al.* (2016)
	No effect	NA	↑	NA	Ueda *et al.* (2013)
		NA	NA	NA	Karley, Emslie-Smith and Bennett (2017)
Effects of aphid feeding on host plant intraradical colonisation by AMF	Increase	NA	NA	NA	Plant species dependent effect Meir and Hunter (2018a)
	No effect	NA	NA	NA	Vannette and Hunter (2014)
		NA	NA	Altered gene expression unlinked to altered aphid performance	Maurya *et al.* (2018)
	Decrease	Measured above ground only		Altered plant volatile profiles aboveground	Babikova *et al.* (2014a)
		NA	NA	NA	Plant species dependent effect; Meir and Hunter (2018a)

[1]Performance refers to growth parameters, including fecundity, development time, growth rate, number of embryos and population number and weight.
[2]Aphid colonisation refers to natural ingress and subsequent proliferation of aphids on host plants.

[3]Increase/decrease refers to an effect on at least one corresponding variable measured in the study. No effect corresponds to no effect recorded for any corresponding variable measured in the study.

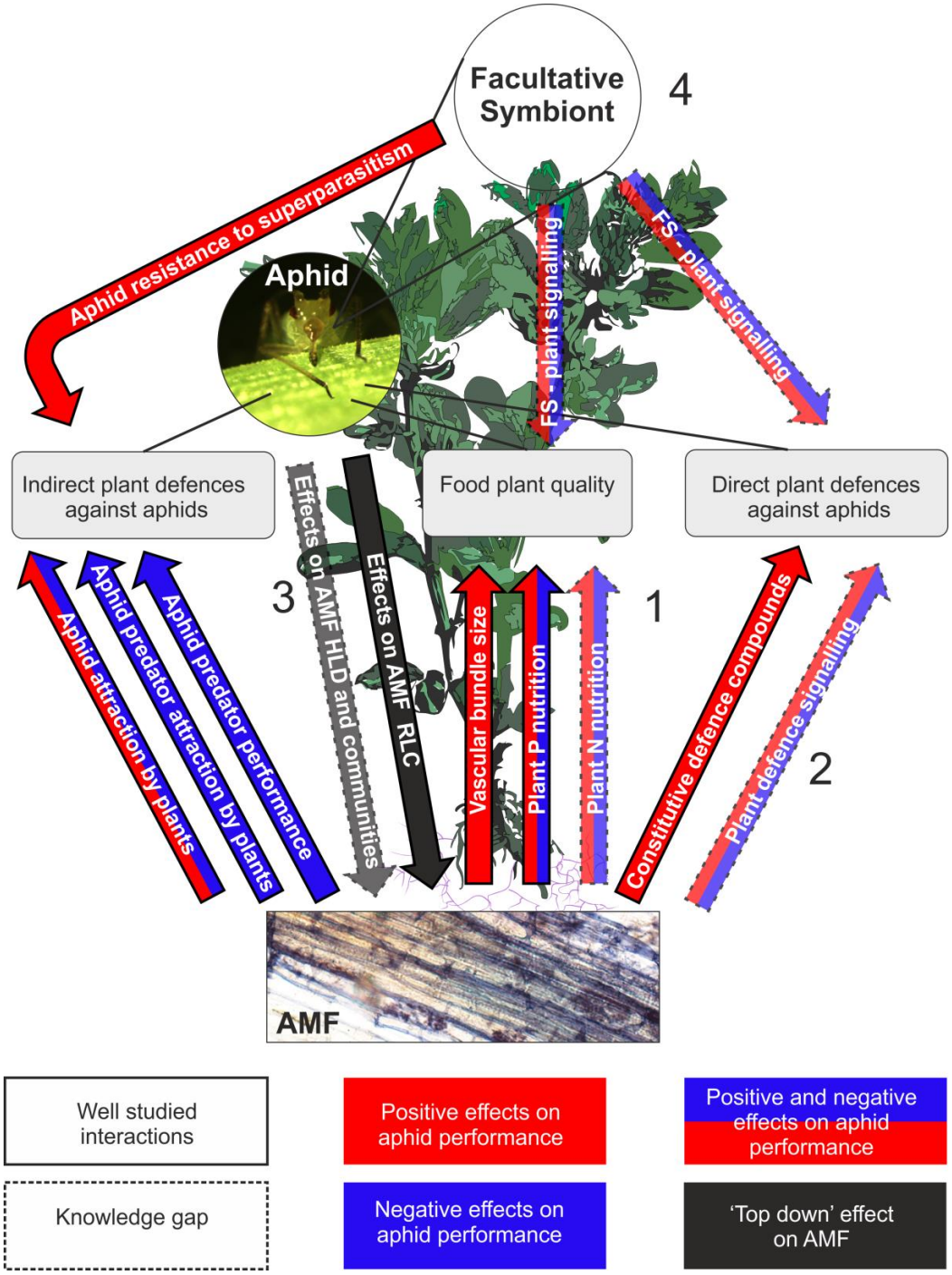

Figure 1. Representation of potential interactions between arbuscular mycorrhizal fungi (AMF) and aphids. Although many such interactions could occur, only well studied interactions (one or more successful investigations into the factors' influence on aphid performance; black outline) and Key Knowledge Gaps (dotted outline, transparent) are shown. Key Knowledge Gaps include 1) The effect of AMF access to nitrogen sources upon aphid and host plant performance and nitrogen status. 2) Multitrophic effects on plant defence signalling and plant defence priming early in the AMF-aphid interaction. 3) The 'top down' effects of aphids on AMF hyphal length density (HLD) and community structure. 4) The impacts of aphid facultative symbionts (FS) upon aphid responses to AMF mediated changes to plant quality and defence. RLC refers to root length colonisation. Image of plant modified from Clipart courtesy FCIT (http://etc.usf.edu/clipart/)

1.2 Multitrophic effects on plant nutrition as a driver of aphid performance

1.2.1 AMF mediated plant nutrient acquisition

AMF have two phases, one inside the root (intraradical mycelium; IRM), and the other outside the root which forms the network of external hyphae in the soil (extraradical mycelium; ERM). Termed the direct P uptake pathway, plants roots directly uptake P in an inorganic form (Pi) as orthophosphate. However, due to the low mobility of Pi in the soil, Pi depletion zones build up close to the plant roots, limiting uptake. Mycorrhizal plants can also take up P via the AMF partner, termed the indirect P uptake pathway; the ERM extends the volume of soil that can be explored beyond that of the plant root and thus extends the Pi depletion zones. Moreover, the finer hyphae can penetrate narrower soil pores than the plant root, further increasing the probability of encountering Pi (Smith *et al.* 2011). The ERM also captures N in inorganic forms such as ammonium (NH_4^+) (Ames *et al.* 1983) and nitrate (NO_3^-) (Tobar, Azcon & Barea 1994), as well as in simple organic forms such as amino acids (Hawkins, Johansen & George 2000). Whilst AMF have no known saprotrophic capabilities (Hodge, Campbell & Fitter 2001; Hodge & Storer 2015), the benefit of increased soil volume exploration by the ERM may aid in the response to spatial-temporal changes in inorganic N availability due to organic N breakdown in the soil (Hodge 2014). AMF could then compete for the newly available N before it is sequestered into the biomass of other soil microbes. The captured N and P is proposed to be transported from the ERM to the IRM as the positively charged amino acid arginine and as negatively charged Pi respectively (Govindarajulu *et al.* 2005; Parniske 2008). Nutrient exchange with the plant occurs at the interface between transporter dense plant derived peri-arbuscular membrane (Pumplin & Harrison 2009) and the arbuscule which it envelops; a highly branched fungal structure diagnostic of the AMF symbiosis that forms in the cortex cells of plant roots (Luginbuehl & Oldroyd 2017). Plant transporters in arbuscule containing cells then take up the P as Pi (Rausch *et al.* 2001; Harrison, Dewbre & Liu 2002; Paszkowski *et al.* 2002), whilst the final transfer of N from the AMF to the plant is less clear (Smith & Smith 2011b). In return, the plant provides the AMF with its source of fixed C in the form of hexose sugars and lipids (Keymer *et al.* 2017; Luginbuehl *et al.* 2017). Mycorrhizal plants can also take up P directly, but in some cases this pathway can be supressed by the AMF, and the AMF take over much of the role of nutrient acquisition (Smith, Smith & Jakobsen 2003; Grace *et al.* 2009). Whether the direct plant uptake pathway of N is affected by AMF colonisation is unknown (Bucking & Kafle 2015; Hodge & Storer 2015).

1.2.2 AMF induced changes to phloem sap accessibility

As well as inducing various biomass responses (Jin *et al.* 2017), AMF colonisation may alter plant physiology, below and above ground (Krishna *et al.* 1981; Yano, Yamauchi & Kono 1996). As phloem feeders, aphids feed by inserting stylets into the sieve elements of plants (Tjallingii & Esch 1993) and it was hypothesised that increases to above ground vascular bundle size during AMF colonisation may aid aphids in locating these sieve elements (Gange & West 1994). Indeed, electrical penetration graphing (EPG) demonstrated increased sieve element location speed and subsequent phloem feeding time of *Sitobion avenae* (F.) feeding on AMF colonised wheat spp. (*Triticum* spp.). In the varieties that were tested, AMF colonisation increased the vascular bundle size of the shared host plant (Simon *et al.* 2017). It is possible that phloem accessibility to aphids may be impacted by the water content of host plants due to changes in turgor pressure and phloem viscosity (Tamburini *et al.* 2018). Plant tissue water content may be altered by AMF, especially under drought and saline conditions (Qiao *et al.* 2012; Aroca *et al.* 2013), but this has yet to be linked to aphid performance (Tomczak & Muller 2018).

1.2.3 AMF induced changes to N availability to aphids

Generally, Hemiptera respond positively to N fertilisation of their host plants (Butler, Garratt & Leather 2012). N is a limiting nutrient in the aphid diet of phloem sap, and the increased performance of aphids including *S. avenae* (Ponder *et al.* 2001; Tamburini *et al.* 2018), *Rhopalosiphum padi* (L.) (Aqueel & Leather 2011; Aqueel & Leather 2012), *Aphis gossypii* Glover (Nevo & Coll 2001), *A. nerii* Boyer de Fonscolombe (Zehnder & Hunter 2008) and *Myzus persicae* (Sulzer) (Rousselin *et al.* 2016) has been reported in response to plant N fertilisation regimes. This has been linked to the concentration of amino acids in the phloem sap (Ponder *et al.* 2000; Nowak & Komor 2010) where the ratio of essential to non-essential amino acids is also a driver of the host plants nutritional quality to aphids (Karley, Douglas & Parker 2002).

However, the role of plant N in the AMF-aphid multitrophic interaction is unclear. In a study investigating the impact of AMF colonisation of *Plantago lanceolata* L. upon *M. persicae* and *M. ascalonicus* Doncaster performance, excessively high aphid mortality occurred on non-AMF plants under high and low N regimes (Gange, Bower & Brown 1999). This did not occur in the AMF treatment counterparts, which suggests that AMF colonisation mediated the quality of the plant for the aphid, but plant tissue N was not measured, so the plant N response remains unknown. Where plant tissue N is measured in AMF-aphid interactions, the response of aphids to plant N status is variable, with aphid performance seemingly associated with (Wurst *et al.* 2004), unresponsive to (Babikova *et al.* 2014b; Williams, Birkhofer & Hedlund 2014) or unlinked to plant N (Gange, Bower & Brown 1999; Hempel *et al.* 2009; Babikova *et al.* 2014a). AMF colonisation of *P.*

lanceolata altered the plants N status, leading to a reduction in the biomass of *M. persicae* nymphs. However, by adulthood, the aphids had recovered this biomass loss, suggesting that at some point during development, aphids can overcome AMF induced changes to plant host N status (Tomczak & Muller 2017; Tomczak & Muller 2018). Several strategies could be employed by aphids to overcome differences in plant tissue N quality: 1) Almost all aphids harbour the primary symbiont *Buchnera aphidicola* which synthesises essential amino acids lacking in the aphid diet (Guenduez & Douglas 2009). As this synthesis may respond to changes in amino acid composition (Douglas, Minto & Wilkinson 2001), *B. aphidicola* could buffer effects of plant N quality. 2) Aphids can change their feeding behaviour depending on host plant quality (Ponder *et al.* 2000; Barrios-SanMartin, Figueroa & Ramirez 2016). Increasing the length of phloem feeding could compensate for lower phloem N concentrations. 3) Aphids can manipulate plant nutrient allocation to better fulfil their dietary requirements (Sandstrom, Telang & Moran 2000; Eleftherianos *et al.* 2006).

Simultaneously measuring plant and aphid N concentration could shed light on the interplay between plant and aphid N status during AMF-aphid interactions. The N status of *A. gossypii* and *Aphis craccivora* Koch was measured simultaneously alongside that of their host plant *Trifolium repens* L. in an AMF-aphid interaction. However, as AMF colonisation did not alter plant above ground N status (Grabmaier *et al.* 2014) whether aphids alter their N uptake on plants of different quality due to AMF colonisation remains unclear (Key Knowledge Gap 1; Figure 1). Furthermore, it is proposed (Wurst *et al.* 2004) that direct competition between plants and AMF partners for available N in the single pot systems commonly used in AMF-aphid interaction studies masks the potential impact of N acquisition by AMF (Leigh, Hodge & Fitter 2009; Thirkell, Cameron & Hodge 2016) upon aphids.

1.2.4 AMF induced changes to P availability to aphids

In contrast to N, a meta-analysis reveals that P fertilisation of the plant host does not lead to an overall significant impact on the performance of Hemiptera (Butler, Garratt & Leather 2012). The performance of *Aphis asclepiadis* Fitch feeding upon common milkweed (*Asclepias syriaca* L.) under varying N and P regimes was positively associated with plant N, and negatively associated with plant P. Analysis of the aphid and plant host N:P ratio revealed that even under the high P regime, the aphid's diet was limited in N, but not in P. It was suggested that the active excretion of P whilst feeding to meet N requirements reduced aphid performance (Tao & Hunter 2012). Furthermore, P fertilisation could indirectly affect aphids by altering plant uptake of other nutrients; for example increased plant host N fertilisation increased *Aphis nerii* Boyer de Fonscolombe performance, but

when combined with plant host P fertilisation plant tissue N levels increased further and aphid performance reduced (Zehnder & Hunter 2009).

In contrast, AMF colonisation increased the P status of plants hosting the potato aphid *Aulacorthum solani* (Kaltenbach), which was proposed to be associated with increases in the aphids performance (Ueda *et al.* 2013). However, similar to N, changes to aphid performance due to AMF colonisation may occur independently of plant P status (Babikova *et al.* 2014a; Tomczak & Muller 2017; Meir & Hunter 2018b). Moreover, the use of P regimes revealed that plant P alone is not responsible for the increased performance of *M. persicae* and *M. ascalonicus* on AMF colonised plants, and plant P fertilisation negated the positive effect that AMF colonisation had on the aphid (Gange, Bower & Brown 1999). It is possible that as increased soil P reduced the proportion of plant roots colonised by AMF, important in the outcome of AMF-aphid interactions (Maurya *et al.* 2018), the effect of AMF upon the aphid was indirectly altered. Thus, whilst evidence suggests that P may not be a limiting factor in the diet of aphids, plant P status could potentially indirectly affect other driving mechanisms in the AMF-aphid interaction.

1.2.5 AMF induced changes to plant Si status and aphid feeding

Silicon (Si) is taken up in plant roots in the form of monosilicic acid (Ma *et al.* 2006). Its transport, distribution and accumulation in above ground plant tissues is thought to reflect a combination of 'passive' processes such as transpiration, and 'active' responses to stimuli such as plant tissue damage (Ma & Yamaji 2015; McLarnon *et al.* 2017). Si is deposited as SiO_2 in abrasive bodies known as phytoliths, which wear down the mandibles of chewing insect herbivores and reduces the assimilation of N in the gut (Massey & Hartley 2009). EPG suggests that phloem feeding insects may also be affected by physical Si defences; brown plant hopper (*Nilaparvata lugens* (Stål)) phloem location time was significantly increased when feeding upon Si fertilised rice (*Oryza sativa* L.) (Yang *et al.* 2017b), whilst there was a trend of *Schizaphis graminum* (Rondani) phloem location time increasing whilst feeding upon Si fertilised wheat (Costa, Moraes & DaCosta 2011). It is possible that increased leaf abrasiveness due to Si deposition could contribute to an insects difficulty to locate the phloem (Yang *et al.* 2018). However, reduced phloem ingestion recorded by EPG suggests that Si fertilisation also induces chemical defences that act upon phloem feeding insects (Costa, Moraes & DaCosta 2011; Yang *et al.* 2017a), and Si fertilisation increases plant defence gene expression and enzyme activity in response to phloem feeding insects (Gomes *et al.* 2005; Yang *et al.* 2017a; Yang *et al.* 2018). AMF colonisation may increase plant root (Kothari, Marschner & Romheld 1990; Anda, Opfergelt & Declerck 2016; Frew *et al.* 2017a) and shoot Si (Garg & Bhandari 2016; Garg & Singh 2018), potentially through increasing plant water uptake (Garg & Bhandari 2016) status, and AMF induced defences against root chewing herbivores may be

dependent upon Si availability (Frew *et al.* 2017b). However, to date the role of plant Si status in AMF-aphid interactions has not been well studied.

1.3 Multitrophic effects on plant defence as a driver of aphid performance

1.3.1 AMF induced changes to plant defences against aphids

As well as altering plant nutritional quality, AMF can have large impacts on plant defence (Mauch-Mani *et al.* 2017). Plants defend against aphid attack using pre-formed physical and chemical constitutive defences that are active even in the absence of herbivory. Due to the associated fitness costs of mounting defences, plants have also developed highly sophisticated attack recognition and response pathways that result in inducible defences activated only upon aphid detection (Wu & Baldwin 2010). Inducible responses against aphids may include: 1) the induction of reactive oxygen species (ROS) (Argandona *et al.* 2001; Lei & Zhu-Salzman 2015) and 2) plant defence related phytohormones (Schwartzberg & Tumlinson 2014; Stewart *et al.* 2016); 3) the subsequent expression of plant defence genes involved in cell wall modification, 4) callose deposition (Saheed *et al.* 2009; Chaudhary *et al.* 2014; Prince *et al.* 2014), 5) the hypersensitive response (Villada *et al.* 2009), 6) pathogenesis related (PR) gene expression (Forslund *et al.* 2000; Moran & Thompson 2001; Pineda *et al.* 2012), 7) secondary metabolite production (Meir & Hunter 2018a), and 8) aphid antagonist attracting volatile compound production (Guerrieri *et al.* 1999).

AMF can influence plant defences both in the absence of the aphid (constitutive defences), or in response to aphid attack (induced defences). For example, AMF colonisation alters the levels of the aphid-toxic secondary metabolite cardinolide in milkweed (*Asclepias*) species (Meir & Hunter 2018a; Meir & Hunter 2018b) to different levels, depending on whether the aphid also shares the plant host. AMF colonisation can also alter the host plants attractiveness to both the aphid itself, as in the case of broad bean *Vicia faba* L. to the pea aphid *Acyrthosiphon pisum* (Harris) (Babikova *et al.* 2014a), and to aphid antagonists, such as the parasitoid wasp *Aphidius ervi* (Haliday) (Guerrieri *et al.* 2004). Moreover, whilst ROS induction and callose deposition have not been investigated in AMF-aphid interactions thus far, the augmentation of these plant defences have been associated with the increased resistance of mycorrhizal plants to other plant pests (Vos *et al.* 2013; Perez-de-Luque *et al.* 2017).

It has been proposed that increased plant nutrition may allow plants to allocate more resources to aphid resistant defences such as secondary metabolites (Salas, Corcuera & Argandona 1990). However, studies using nutrient regimes to minimise the difference in nutrient acquisition between AMF colonised and non-colonised plants reveal that AMF

induced alterations to plant defences may also occur irrespective of plant nutrient status (Fritz *et al.* 2006; Liu *et al.* 2007; Babikova *et al.* 2014b).

1.3.2 Plant recognition and response to aphid herbivory

Evidence suggests that the recognition of aphids by plants share many parallels between the multi-layered 'zig-zag' model suggested for plant recognition of microbial pathogens (Nalam, Louis & Shah 2018). The first phase of this model includes basal immunity, in which conserved molecular patterns from the attacker are perceived by pattern recognition receptors (PRRs) on the extracellular plasma membrane of plant cells (Zipfel 2014), sometimes alongside co-receptors (Chaudhary *et al.* 2014). This leads to downstream plant defence signalling processes and is thus also referred to as pattern triggered immunity (PTI) (Zipfel 2014). PRRs and thus PTI may be activated by herbivore, damage to plant tissue, or microbial associated molecular patterns (H/D/MAMPs). Whole plant extracts of aphids elicit PTI like responses (Prince *et al.* 2014) and it is proposed that chitin from the aphid stylet may act as a HAMP, similar to chitin MAMPs (Jaouannet *et al.* 2014). Moreover, many aphid salivary proteins show potential to be HAMPs as they induce plant defence responses (Elzinga, De Vos & Jander 2014). Although research upon DAMPs has focused upon leaf chewing herbivores (Acevedo *et al.* 2015), MAMPs may play a large role in plant recognition of aphids. Microbial proteins are present in aphid honeydew (Sabri *et al.* 2013) and the chaperonin GroEL, which induces PTI responses in the host plant, is derived from the aphid's primary symbiont *B. aphidicola* (Chaudhary *et al.* 2014).

During the second phase of the 'zig zag' model, aphids supress or interfere with the plant's recognition and response to herbivory. Aphids secrete two types of saliva during the probing and feeding process. Gelling saliva may shield the aphid stylet from plant perception and also seal plant cell puncture sites incurred during aphid probing (Abdellatef *et al.* 2015; Will & Vilcinskas 2015). Watery saliva injected into the vascular tissue of the plant contains proteins that may bind calcium, thus reducing the signal for the plant to produce protein plugs and callose to occlude sieve tubes (Will *et al.* 2007). Moreover, studies supressing the expression of salivary proteins in aphids and expressing them in plant hosts have identified a number of aphid salivary proteins with potential roles as 'effectors' that supress plant immune responses (Kaloshian & Walling 2016).

In the third phase of the 'zig zag' model, plants may employ resistance (R) proteins; receptors that recognise these pest effectors and confer 'gene for gene' or effector triggered immunity (ETI). Although the identity of the aphid effectors recognised by plant R genes are yet to be elucidated (Kaloshian & Walling 2016), R genes effective against aphids have been cloned in a handful of plant species (Rossi *et al.* 1998; Wroblewski *et*

al. 2007; Villada *et al.* 2009) and ETI results in a faster and more efficient downstream signalling and defence response than PTI (Villada *et al.* 2009). However, there is much crossover between the PTI and ETI pathways and many defence responses induced by ETI and PTI in response to aphid herbivory are shared (Goggin 2007; Villada *et al.* 2009).

1.3.3 Modulation of plant defence signalling in aphid-plant interactions

Fine-tuned by other signalling compounds such as abscisic acid, ethylene, and gibberellic acid, the plant phytohormones salicylic acid (SA) and jasmonic acid (JA) play major roles in determining the specifics of the plant defence response. SA signalling is most commonly induced by, and associated with, the control of biotriophic pathogens that feed on live hosts, whereas JA induced pathways are associated with necrotrophic pathogens and leaf chewing herbivores (Berens *et al.* 2017). Low levels of mechanical damage via intracellular feeding and manipulation of plant defences create an intimate association with the plant, which allows aphids to keep phloem cells alive. This may explain the mirroring of plant defence signalling responses to aphids and biotrophic pathogens (i.e. the upregulation of SA based defences (Jaouannet *et al.* 2014)).

Whilst SA signalling can be associated with increased resistance to aphids (Moran & Thompson 2001; Thaler, Agrawal & Halitschke 2010; Kerchev *et al.* 2013). SA signalling can also be associated with increased aphid performance (Jaouannet *et al.* 2014). Exogenous application of JA (Cooper & Goggin 2005; Gao *et al.* 2007; Haas *et al.* 2018) and the upregulation of JA defences (Losvik *et al.* 2017) can be detrimental to aphids. With some exceptions, JA and SA signalling are commonly antagonistic (Thaler, Humphrey & Whiteman 2012) and it is thus proposed that aphids may induce SA signalling to repress JA pathway defence induction in their plant host (Giordanengo *et al.* 2010). Indeed, *A pisum* feeding and honeydew deposition increased SA signalling in *V. faba*, and reduced JA responses induced by mechanical damage at a site distal to the aphid (Schwartzberg & Tumlinson 2014). It should be noted however, that the performance of *A. pisum* specialised to different legumes were highest on plants where both SA and JA defences were suppressed (Sanchez-Arcos *et al.* 2016), suggesting that both SA and JA pathway defences may be detrimental to aphids.

1.3.4 Modulation of plant defence signalling in AMF-aphid interactions, and plant defence priming

AMF also influence plant defence signalling. It is currently proposed (Cameron *et al.* 2013; Perez-de-Luque *et al.* 2017) that the perception of AMF associated molecules, potentially lipochitooligosaccharides (Mauch-Mani *et al.* 2017), triggers downstream signalling processes within plant roots that the AMF must subsequently supress (Jung *et al.* 2012). This could lead to AMF constitutively influencing plant defences. However, AMF can also

augment plant resistance without inferring the large energetic costs of constitutive defence induction. During (plant defence) priming, the interaction with a priming stimulus, such as a beneficial microbe or priming inducible chemical, confers a primed state to the plant. Upon entering this state, the plant may accumulate small amounts of defence related metabolites, but otherwise a defence response is only induced after pest attack. This defence response to an attacker is stronger and/ or quicker than in an un-primed plant (Martinez-Medina *et al.* 2016) (Figure 2). Priming is thus associated with increased basal immunity (Perez-de-Luque *et al.* 2017).

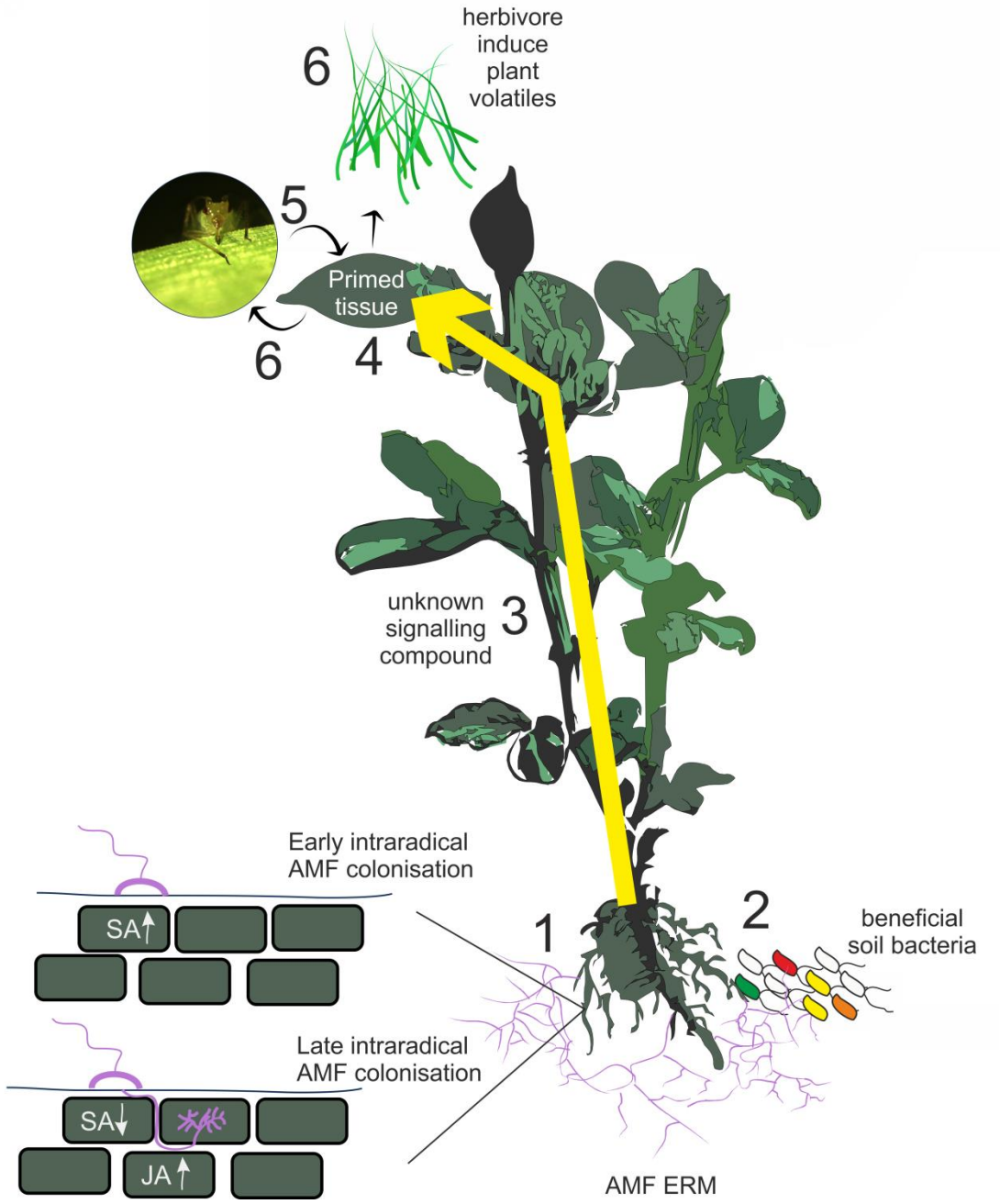

Figure 2. Figure representing the proposed model of plant tissue 'priming' against aphids by AMF. 1) Perception of AMF associated molecules during early AMF colonisation events induces SA defence signalling in the host plant, leading to the priming of SA defences. Suppression of SA signalling by AMF, and upregulation of other defence pathways later in the colonisation event, alongside 2) the recruitment of beneficial soil bacteria to the plant root by AMF leads to the priming of other defence pathways including JA. 3) An as yet unknown signalling molecule is transported systemically in the plant, resulting in 4) primed plant tissue with slightly induced defence related compounds. 5) Enhanced perception of aphid feeding in primed material. 6) Augmented direct and indirect defence responses against the aphid attacker. Image of plant modified from Clipart courtesy FCIT (http://etc.usf.edu/clipart/).

A functional priming response may require an intact JA signalling pathway (Song *et al.* 2013; Nair *et al.* 2015), although priming during MIR can augment both SA and JA defences (Gallou *et al.* 2011; Hao *et al.* 2012; Nair *et al.* 2015; Song *et al.* 2015). It is proposed (Cameron *et al.* 2013; Perez-de-Luque *et al.* 2017) that the upregulation of the SA defence pathway during AMF colonisation primes SA based defences. The suppression of these defences by AMF, and the recruitment of beneficial bacteria by AMF to the plant root may then result in the induction and priming of other defence pathways, including the JA pathway (Cameron *et al.* 2013). It was recently demonstrated that the priming of plant defences in MIR is further augmented in the presence of rhizosphere bacteria, supporting this model (Perez-de-Luque *et al.* 2017), and grafting experiments suggest a currently unknown signal is translocated from the roots to above ground plant tissues to induce a primed state (Mora-Romero *et al.* 2015).

The plant defence signals involved in priming depend on plant and pest identity (Mauch-Mani *et al.* 2017). The only study reporting plant defence signalling in priming against an above ground arthropod (the leaf chewing *Helicoverpa armigera* (Hübner)), associates MIR with augmented JA defences (Song *et al.* 2013). Despite recent efforts (Maurya *et al.* 2018), if, and which, defence pathways are associated with MIR against phloem feeding insects is currently unclear: *A. pisum* performance was reduced in certain contexts on *Medicago truncatula* Gaertner colonised by AMF, but this could not be linked to plant defence gene expression. It was proposed that as plant defence gene expression was measured 7 days post aphid addition, key interactions between AMF and aphids upon plant defence signalling could have been missed.

It is also possible that the modulation of plant defences by a beneficial microbe will lead to increased susceptibility to a pest; colonisation by the plant growth promoting rhizobacteria *Pseudomonas fluorescens* Migula augments *Arabidopsis thaliana* (L.) JA pathways, but supresses abscisic acid pathways leading to increased susceptibility to the cabbage aphid *Brevicoryne brassicae* (L.) (Pineda *et al.* 2012). As aphids manipulate plant defence signalling pathways in host plants, it is possible that AMF colonisation could augment this manipulation, leading to induced susceptibility in AMF-aphid interactions. Whether priming occurs in AMF-aphid interactions, as well as the interplay of JA and SA signalling underpinning this phenomenon require elucidation (Key Knowledge Gap 2; Figure 1).

1.4 Impacts of aphids upon AMF

Via the shared host plant, above-ground insect herbivores can indirectly impose ‘top down’ effects in multitrophic interactions, altering the developmental or community structure characteristics of below ground organisms (Ba *et al.* 2012; Kong *et al.* 2016; Hoysted *et al.* 2018). If this developmental or community characteristic is a factor that

modulates the ‘bottom up’ multitrophic interaction, this could feedback into the performance of the above ground pest and an above-below ground feedback loop occurs (Kostenko *et al.* 2012). Thus, it is important to elucidate the effects of aphids upon AMF sharing the host plant to predict whether such feedback loops could occur. Only the impact of aphid herbivory upon intraradical colonisation of the shared host plant by AMF has been investigated so far in AMF-aphid interactions. Similar to bottom up effects of AMF upon aphids, aphid infestation of the shared host plant may reduce, have no effect on, or increase AMF intraradical colonisation of the host plant (Table 1). As the level of intraradical colonisation by AMF may modulate the effect of AMF upon aphids (Maurya *et al.* 2018; Meir & Hunter 2018b), potentially due to the suppression of high AMF colonisation levels by plant defences leading to different levels of plant defence priming (Meir & Hunter 2018b), this could induce a feedback loop. Again, the proposed mechanisms underlying these ‘top down’ effects can be broadly split into alterations to plant nutrition and defence (van Dam & Heil 2011).

Aphid herbivory can reduce the concentration of sugars exudated from the roots (Hoysted *et al.* 2018). The C limitation hypothesis (Wallace 1987) proposes that the removal of fixed C and photosynthetic material from the plant by above ground herbivores will reduce the C available to below ground organisms, impairing colonisation of the host plant. However, akin to bottom up effects of AMF on herbivores, an opposite effect may occur, and AMF colonisation can increase, at least under moderate herbivory of the host plant (Wamberg, Christensen & Jakobsen 2003; Barto & Rillig 2010). It is proposed that under intermediate levels of herbivory, the host plant may allocate more resources below ground in order to increase nutrient uptake to regrow above ground tissue (Wamberg, Christensen & Jakobsen 2003).

Plant defence signalling may also play a role; infestation by the phloem feeding whitefly (*Bemisia tabaci* (Genn.)) induces SA, and JA/ET dependent signalling pathways in pepper (*Capsicum annuum* L.) roots (Park & Ryu 2014), and chemical elicitation of plant defence pathways above ground can reduce AMF intraradical colonisation (Faessel *et al.* 2010; de Roman *et al.* 2011). As SA signalling is supressed in the late stages of plant root AMF colonisation, possibly to maintain the symbiosis (Blilou, Ocampo & Garcia-Garrido 1999), it is proposed that increased SA signalling due to above ground phloem feeding may contribute to reduced AMF colonisation (Babikova *et al.* 2014a). Moreover, AMF signal the presence of above ground attackers (Song *et al.* 2010; Song *et al.* 2014) including aphids (Babikova *et al.* 2013a; Babikova *et al.* 2013b) to neighbouring plants via connected extraradical hyphae, suggesting messages of above ground attack are passed between the plant and AMF partner. So far, aphid feeding has been linked to reduced gibberellic

acid biosynthesis genes in *M. truncatula* colonised with high levels of AMF, but this did not result in reduced AMF RLC (Maurya *et al.* 2018).

The C limitation hypothesis has been extended to encompass AMF structures outside of the root, i.e. ERM, as well as the diversity of the AMF community colonising the host plant (Gange 2007). As the density of AMF hyphae are directly related to the transport of nutrients to the plant host (Barrett, Campbell & Hodge 2014), and the community of AMF colonising the plant root may also determine the level of nutrient uptake (Manoharan *et al.* 2017; Jiang *et al.* 2018) and induced pest resistance a plant receives from the symbiosis (Pozo *et al.* 2002; Sikes, Cottenie & Klironomos 2009; Malik, Dixon & Bever 2016), changes to these AMF characteristics could influence the outcome of AMF-aphid interactions. However, contrasting AMF communities due to different agricultural management practices did not impact *R. padi* performance on barley (Williams, Birkhofer & Hedlund 2014).

It is proposed that increased C availability will allow less competitive AMF species to colonise the plant root, increasing AMF species richness (Gange 2007). However, as AMF communities shaped by above ground herbivory by large vertebrates provide increased defoliation tolerance in perennial bunchgrass (*Themeda trianda* Forssk)(Gonzalez *et al.* 2018), it is suggested that herbivory could cause plants to recruit communities effective in providing improved herbivore tolerance. Insect herbivory may alter the ectomycorrhizal (Gehring & Bennett 2009), non-mycorrhizal fungi (Kostenko *et al.* 2012) and bacterial communities (Bjornlund *et al.* 2006; Yang *et al.* 2011; Park & Ryu 2014; Kong *et al.* 2016) associated with plants below ground, although the effect of phloem feeders upon both AMF ERM and community structure remains to be elucidated (Key Knowledge Gap 3; Figure 1).

1.5 Facultative aphid symbionts in AMF-aphid interactions

As previously discussed, almost all aphids carry the obligate bacterial primary symbiont *B. aphidicola,* integral for host survival by synthesising essential amino acids otherwise attained in low quantities from phloem sap (Douglas 2006). Although *B. aphidicola* resides in specialised aphid cells termed bacteriocytes (Moran *et al.* 2005) proteins derived from the primary symbionts genome trigger plant defences against the aphid (Chaudhary *et al.* 2014) and thus potentially play a major role in AMF-aphid interactions. Aphids may also carry single, or simultaneous ‘infections’ of multiple (secondary) bacterial facultative symbionts (FS) (Zytynska & Weisser 2016; Guo *et al.* 2017) that, whilst not integral to host survival, may lead to mutualistic benefits by providing protection against a range of biotic and abiotic stresses (Montllor, Maxmen & Purcell 2002; Oliver *et al.* 2003; Scarborough, Ferrari & Godfray 2005; Heyworth & Ferrari 2015). Whether this extends to the protection

of aphids from possible detrimental effects of shared host plant AMF colonisation is currently unclear (Bennett *et al.* 2016). Crucially, for AMF-aphid interactions the harbouring of FS may influence interactions with plant defences (Frago *et al.* 2017). Moreover, FS may influence host plant range (Tsuchida, Koga & Fukatsu 2004; Tsuchida *et al.* 2011; Henry *et al.* 2013), which in leaf chewing herbivores is associated with insect symbiont mediated suppression of plant defence (Chung *et al.* 2017). The suppression of plant defences by FS is also important in the performance of aphids on different plant hosts (Sanchez-Arcos *et al.* 2016).

FS may interfere in indirect plant defence interactions by protecting the aphid host against aphid antagonists, including parasitoid wasps (Oliver *et al.* 2003; Heyworth & Ferrari 2015). In *Solanum* spp. the potential interaction between the below ground plant symbiont AMF and the above ground aphid FS *Candidatus* Hamiltonella defensa Moran *et al.* (henceforth referred to as *H. defensa*) was investigated in relation to the attack of aphids by the parasitoid wasp *A. ervi*. Whilst wasp attack and emergence was highest upon aphids reared on AMF colonised plants, the presence of *H. defensa* did not modify the interaction (Bennett *et al.* 2016), potentially due to *H. defensa* strain context dependency. The level of protection *H. defensa* provides for the aphid depends on the strain of the symbiont (Oliver, Moran & Hunter 2005) and this is associated with the type of bacteriophage (APSE) carried by *H. defensa,* which codes for eukaryotic toxins that may disrupt the development of the parasitoid egg (Oliver *et al.* 2009).

The modulation of *direct* plant defence signalling by phloem feeding associated FS has only been investigated in whitefly with *H. defensa* thus far, and whitefly infection with *H. defensa* supressed host plant JA signalling, leading to increased whitefly performance (Su *et al.* 2015). As FS infection of *A. pisum* attenuates aphid antagonist attracting volatile compounds produced by the plant during aphid feeding (Frago *et al.* 2017), this suggests that aphid associated FS can also interact with plant defences. Thus, it is possible that aphid FS add another level to interactions with plant host defence in AMF-aphid interactions.

Under certain circumstances, harbouring FS may be detrimental to the aphid. Similar to AMF-plant host symbiotic interactions, infection of an aphid by a FS incurs a cost to the host that may become apparent under circumstances where the benefit of the symbiosis is not required. This may manifest as reduced aphid performance under benign conditions, but may be dependent on aphid and FS genetic contexts (Vorburger & Gouskov 2011). Infection of aphids with FS can supress, or induce levels of the primary symbiont *B. aphidicola* (Sakurai *et al.* 2005; Li *et al.* 2018) potentially altering the ability of aphids to cope with poor N quality hosts (Zytynska & Weisser 2016). Thus aphid FS could influence plant nutrient driven interactions between AMF and aphids. Furthermore,

although FS can modulate plant defences in favour of the insect host (Su *et al.* 2015; Frago *et al.* 2017), the reduced root biomass of *Solanum* spp. fed upon by *H. defensa* carrying *M. euphorbiae* (Hackett, Karley & Bennett 2013; Bennett *et al.* 2016) is a common feature in plant stress responses (Guiguet *et al.* 2016). It is currently unclear whether, like the primary symbiont *B. aphidicola* (Chaudhary *et al.* 2014), FS produce molecules that elicit plant defence responses. In summary, as protective phenotypes may only become apparent in certain aphid genotype-FS strain combinations, multiple FS strains need to be tested in AMF-aphid interactions. Moreover, the role of shared plant host nutrition and defence in the tri-trophic interactions between AMF, aphids and FS requires elucidation (Key Knowledge Gap 4; Figure 1).

1.6 Thesis aims

This work aims to investigate the Key Knowledge Gaps identified in understanding AMF-aphid interactions, and the implication of aphids carrying their own symbionts for this interaction (Figure 1). Using AMF-plant-aphid systems suited to the investigation of each knowledge gap, the specific objectives of this project were:

1. To investigate the impact of AMF delivered N upon plant performance, alongside the performance of the plant's associated aphid herbivore.
2. To investigate the interplay between JA and SA pathway defence signalling in AMF-aphid interactions.
3. To investigate the impact of aphid infestation of the shared plant host upon extraradical AMF structures and AMF communities.
4. To assess whether or not aphid infection by FS and FS strain type influences the response of aphids to AMF colonisation of the shared plant host.

2 Impacts of genotype upon AMF, barley and *Sitobion avenae* (F). interactions

Disclaimer: This chapter includes work from James Wilson regarding the first experiment outlined. JW Designed, set up and harvested the first experiment detailed. TW analysed the samples and data, and wrote the report. The second and third experiments outlined were designed, carried out, analysed and written by TW.

2.1 Introduction

The majority of the world's calorific intake is supported by agricultural systems supplemented with high inputs of inputs of nitrogen (N), phosphorus (P) and pesticides (Paungfoo-Lonhienne *et al.* 2012). However, the need to improve crop yields further raises concerns of environmental sustainability (FAO 2011). P fertiliser production is resource limited (van Vuuren, Bouwman & Beusen 2010; van de Wiel, van der Linden & Scholten 2016), and whilst there is promise for N fertiliser production to become less detrimental to the environment (Kyriakou *et al.* 2017), the application of fertilisers can directly lead to environmental pollution (Pretty *et al.* 2005). Furthermore, concerns of non-target effects of pesticides (Antwi & Reddy 2015; van der Sluijs *et al.* 2015) and the development of plant pest resistance (Foster *et al.* 2014) suggests that reducing pesticide applications is important in achieving agricultural sustainability.

Multitrophic interactions between crops and beneficial microorganisms can have implications for nutrient uptake and resistance to crop pests, and thus could reduce fertiliser and pesticide inputs. Such interactions are also influenced by the genotypes of the organisms concerned. Thus, it can be informative to explore different genotypes when creating model systems for investigating multitrophic interactions. For example, a pest's genotype influences its fecundity (Zytynska & Preziosi 2011), interactions with host plants (Zytynska *et al.* 2016), host plant associated microbes (Tetard-Jones *et al.* 2007) and resistance to biotic and abiotic stresses (Lukasik *et al.* 2011; Lukasik *et al.* 2013; Malloch *et al.* 2014). Similarly, genetic variation influences a crop variety's ability to take up and use nutrients (Beatty *et al.* 2010), resist pests (Dreiseitl 2015), and interact with soil microbes (Boyetchko & Tewari 1995).

The process of domestication, for example, has led to genotypic variation in crops which may alter how crops interact with beneficial soil microbiota (Leff *et al.* 2017; Perez-Jaramillo *et al.* 2017; Martin-Robles *et al.* 2018). Two thirds of vascular plants form symbioses with arbuscular mycorrhizal fungi (AMF) (Hughes *et al.* 2008). The plant provides fixed carbon (C) to AMF partners, and this below ground obligate symbiont may deliver N and P to the plant host (Ames *et al.* 1983; Harrison, Dewbre & Liu 2002; Leigh, Hodge & Fitter 2009) and confer resistance to biotic (Wurst *et al.* 2004; Campos-Soriano,

Garcia-Martinez & San Segundo 2012) and abiotic stresses (Zhang *et al.* 2005; Talaat & Shawky 2012). Moreover, AMF can reduce the effects of pollution associated with fertiliser inputs (Bender, Conen & van der Heijden 2015; Cavagnaro *et al.* 2015; Storer *et al.* 2017). The capacity of plants to form and control this symbiosis is associated with the proportional root length colonised (RLC) by the fungus (An *et al.* 2010), and in certain crops, RLC may be reduced in modern cultivars comparison to ancestors, landraces or older varieties (An *et al.* 2010; Lehmann *et al.* 2012; Xing *et al.* 2012; Turrini *et al.* 2016; Salloum, Menduni & Luna 2018). In some cases (Lehmann *et al.* 2012; Chu *et al.* 2013), but not all (Ellouze *et al.* 2016; Leiser *et al.* 2016), RLC may be linked to a variety's ability to derive a nutritional or growth benefit from the symbiosis. Moreover, interactions between AMF and above ground pests are proposed to be more likely to occur at higher levels of colonisation (Tomczak & Muller 2017).

Barley (*Hordeum vulgare* L.) is the UK's second most cultivated crop (DEFRA 2017). After a complex domestication process occurring around 10,000 years ago (Tanno & Willcox 2012; Pankin & von Korff 2017), barley has been cultivated under relatively low agrochemical inputs until the Green Revolution (circa 1940s-1970s) where cereals were selectively bred under markedly increased nutrient and pesticide inputs. The breeding of crop varieties under these conditions is proposed to have removed the need for the nutritional and bio-protective roles of the AMF symbiosis, leading to the impromptu selection of plants with reduced capacity to interact with AMF (Hetrick, Wilson & Cox 1992; Zhu *et al.* 2001). Moreover, it is proposed that trade-offs between breeding for fungal pathogen resistance and AMF recruitment may occur, as there is overlap in the plant signalling pathways AMF and fungal pathogens rely on to colonise the host plant (Ruiz-Lozano, Gianinazzi & Gianinazzi-Pearson 1999; Guimil *et al.* 2005; Jacott, Murray & Ridout 2017). For example, barley varieties carrying the powdery mildew resistant 'mlo' mutation may have reduced capacity for AMF colonisation (Ruiz-Lozano, Gianinazzi & Gianinazzi-Pearson 1999), and maize (*Zea mays* L.) varieties resistant to fungal pathogens exhibit reduced RLC, although root architecture was also different amongst these maize varieties (Toth *et al.* 1990). As AMF may preferentially colonise lateral roots, changes to root architecture could alter RLC (Schmidt, Bowles & Gaudin 2016).

However, in certain crop species, modern varieties may not be reduced in RLC (Koide *et al.* 1988; Leiser *et al.* 2016), and in wheat (*Triticum* spp) and maize both increases and decreases are both reported (Hetrick, Wilson & Cox 1993; An *et al.* 2010; Chu *et al.* 2013; Ellouze *et al.* 2016; Aguilar *et al.* 2017; Ercoli *et al.* 2017). This lack of a general trend is reflected in a study investigating 27 crop species and their ancestors (Martin-Robles *et al.* 2018). The effect of modern breeding practices upon barley RLC is also mixed (Zhu, Smith & Smith 2003; Castellanos-Morales *et al.* 2011; Martin-Robles *et al.* 2018).

However, these studies only investigated one modern barley variety. As barley genotypes may intrinsically differ in RLC (Boyetchko & Tewari 1995), and RLC may differ depending on growth conditions, including soil type, water capacity, nutrition and pot size (Zhu, Smith & Smith 2003; Chu *et al.* 2013; Ryan *et al.* 2016; Martin-Robles *et al.* 2018) comparisons between multiple genotypes need to occur in the same study.

One benefit of interactions with AMF is the potential to reduce the performance of aphids sharing the same host plant (Guerrieri *et al.* 2004; Wurst *et al.* 2004; Hempel *et al.* 2009). The English grain aphid (*Sitobion avenae* (F.)) is an important economic pest (Larsson 2005; Ramsden *et al.* 2017) which feeds upon the phloem of barley, most often from the leaves or the emerging grain (Blackman & Eastop 2000). However, AMF colonisation may have no effect on aphid performance (Williams, Birkhofer & Hedlund 2014; Bennett *et al.* 2016; Karley, Emslie-Smith & Bennett 2017), or even increase it (Gange & West 1994; Simon *et al.* 2017). Thus, the genetic or environmental contexts that cause this variation in response require investigation.

The types of defences induced by barley in response to aphids (Forslund *et al.* 2000; Argandona *et al.* 2001; Casaretto, Zuniga & Corcuera 2004; Saheed *et al.* 2009) have been reported to be augmented by AMF colonisation in other plant- pest species interactions (Song *et al.* 2015; Nath *et al.* 2016; Perez-de-Luque *et al.* 2017). This augmentation of plant defence is proposed to arise via the modulation of plant defence pathways during the establishment of AMF colonisation (Cameron *et al.* 2013; Perez-de-Luque *et al.* 2017), in which a functioning jasmonic acid (JA) plant defence pathway can be integral for the increased protection (Song *et al.* 2013; Nair *et al.* 2015). The profile of plant defence signalling pathways induced during AMF colonisation is dependent upon the fungal species: across distantly related plant species, colonisation by the AMF *Funneliformis mosseae* (Nicolson & Gerd.) Walker & Schüßler upregulates JA pathways more than *Rhizophagus irregularis* (Schenck & Smith) Walker & Schüßler (Fernandez *et al.* 2014). This is proposed (Fernandez *et al.* 2014) to be reflected in the increased bioprotective effect of *F. mosseae* over *R. irregularis* (Pozo *et al.* 2002; Mustafa *et al.* 2016).

AMF could also affect aphids by altering plant nutrition. AMF can deliver N and P to the plant host, which can also depend on the AMF species (Jansa, Smith & Smith 2008; Grace *et al.* 2009; Leigh, Hodge & Fitter 2009). AMF delivery of P can have large impacts on plant growth (Smith & Smith 2011b), whilst N is a limiting nutrient in the diet of aphids (Butler, Garratt & Leather 2012). Although it is unclear whether AMF species differ in their ability to increase plant silicon (Si) uptake, plants colonised by AMF can take up more Si. Although the mechanism of increased uptake is unclear, this may increase plant resistance to below ground herbivores (Frew *et al.* 2017a). Si fertilisation of host plants

can reduce *S. avenae* performance (Dias *et al.* 2014), though not always (Massey, Ennos & Hartley 2006).

Moreover, genetic dependencies can occur at the level of the aphid: *S. avenae* performance on a given host plant can be influenced by the aphid genotype (Debarro *et al.* 1995; Zytynska & Preziosi 2011), potentially as cereal aphid genotypes induce unique defence gene related transcription profiles in host plants (Zaayman, Lapitan & Botha 2009; Liu *et al.* 2011; Zytynska *et al.* 2016). As aphid genotype influences interactions with plant defences, this could determine how aphids respond to AMF colonisation of the host plant.

The aim of the series of experiments in this chapter was to identify the most suitable model system for investigating AMF-barley-*S. avenae* interactions for use in subsequent experiments. This study aimed to investigate whether modern varieties of barley differ in their capacity to form symbioses with AMF compared to older and less selectively bred varieties (Experiment 1). Two separate studies (Experiment 2 and 3) aimed to investigate the impact of AMF colonisation of barley upon *S. avenae* performance with two AMF species and aphid genotypes. Specifically, the following hypotheses were tested: 1) a) modern varieties of barley will have reduced RLC compared to older varieties and landraces, due to selection for yield under high agricultural inputs and fungal pathogen resistance, and b) RLC will be associated with plant root size. 2) AMF colonisation of barley will reduce the performance of *S. avenae* but only when the plant is colonised by *F. mosseae*, due to its tendency to upregulate JA signalling more so than *R. irregularis* during host plant colonisation. 3) AMF colonisation will increase plant N, P and Si uptake depending on the AMF species colonising the plant. 4) Aphid performance will depend on aphid genotype.

2.2 Materials and methods

2.2.1 AMF colonisation of modern barley varieties, traditional varieties and landraces (Experiment 1)

Barley varieties were selected due to having either been bred under modern agricultural practices (modern cultivars), or under traditional cultivation methods (traditional varieties or landraces) (Table 2).

Table 2. Modern barley cultivars, or traditional varieties and landraces used in the arbuscular mycorrhizal colonisation screen

Barley variety	Cultivar/landrace	Place of origin	Date of origin	Reference	Seed provider
Rum	Traditional variety	Jordan	NA	(Samarah *et al.* 2009; Shakhatreh *et al.* 2010)	
Arta	Landrace	Syria	NA	(Rollins *et al.* 2013)	Max Planck Institute
Bere	Landrace	UK	8th century	(Martin, Chang & Wishart 2010)	Max Planck Institute
Chevalier	Landrace	UK	19th century	(Beaven 1936)	
Galt	Cultivar	Canada	1970's	(Wooding *et al.* 1982)	USDA
Optic	Cultivar	UK	1996		Syngenta
Xanadu	Cultivar	Germany	2003		SAATEN-UNION
Quench	Cultivar	UK	2006		Syngenta
Irwina	Cultivar	UK	2014		KWS

KWS UK Ltd., Hertfordshire, UK. SAATEN-UNION GmbH, Isernhagen, Germany. Max Planck Institute for Plant Breeding Research, Cologne, Germany. United States Department of Agriculture, Washington, D.C., United States. Syngenta UK Limited, Fulbourn Cambridgeshire, UK

Barley seeds were surfaced sterilised in 5 % (w/v) sodium hyperchlorite solution and allowed to germinate on filter paper. After five days, one germinated seed was transferred to 800 mL of autoclaved (121°C for 10 minutes during 90 minutes run cycle) 1:1 (v:v) sand: Agsorb® (Oil-Dri, Cambridgeshire, UK) mixed with 50 g of single species *R. irregularis* inoculum (Plantworks UK, Ltd. Kent, UK), added as colonised *Trifolium pratense* L. and *Plantago lanceolata* L. roots (grown in 1:1 (v:v) sand:Agsorb®). *R. irregularis* is a generalist coloniser and for this reason has been used in previous RLC screening studies (Martin-Robles *et al.* 2018). The standardised non-soil growth media was used to reduce bias of plant growth due to plant variety preference for a particular soil type. *P. lanceolata* seeds were surface sterilised and planted directly into the growth medium at 10 seeds per pot in a separate treatment to assess mycorrhizal inoculum health by comparing their RLC in the current experiment with that of a previous study.

Seven replicates of each treatment were set up in a randomised block design in a lit, (400 Watt high-pressure sodium lights), heated glasshouse with a 16 hour day length. Plants were fed twice weekly with 50 mL nutrient solution (Thornton & Bausenwein 2000) modified to contain 10 % of the original P to encourage AMF colonisation (Leigh, Hodge & Fitter 2009), and were watered with dH_2O if and when necessary. Harvest occurred 35 days post seedling transfer to pots, where root, leaf and stem fractions were separated and dried in a 70°C oven for at least 72 h. Any grain was included with stem biomass and sub sections of fresh plant roots were stored in 40 % ethanol to await acetic acid-ink staining (Vierheilig *et al.* 1998) with the following modifications: Roots were thoroughly rinsed in dH_2O and subsequently incubated for 45 mins in 10 % KOH at 70°C. After, the roots were rinsed with dH_2O and stained for 30 mins with 5 % Pelikan® brilliant black ink and 5 % acetic acid in 90 % dH_2O. The gridline intercept method was used to assess RLC, arbuscules and vesicle frequency (Hodge 2003) under 200 x magnification using a Nikon eclipse 50i upright microscope (Nikon UK ltd, Surrey, UK). Root staining of one replicate of Xanadu, Bere, Irwina and Chevalier barley varieties were unsuccessful, and these replicates were removed from analysis. Furthermore, plant material was lost during the drying process, resulting in (for root dry weight and root weight ratio (RWR) only); 6 replicates of Quench, Rum, Galt; 5 replicates of Xanadu, Bere, optic, Chev, Arta; and 4 replicates of Irwina barley varieties. RWR was calculated as the proportion of plant biomass allocated to the root (root dry weight/ total plant dry weight).

2.2.2 Effect of AMF species and aphid genotype in AMF-barley-*S. avenae* interactions (Experiment 2)

A highly mycorrhizal cultivar at five weeks post AMF inoculation (Xanadu; Saaten Union GmbH, Isernhagen, Germany) was selected to test the effect of AMF colonisation on *S. avenae* performance. Plants were colonised by *R. irregularis*, *F. mosseae* or no AMF and

were free of aphids or infested with one of two genotypes of *S. avenae* in a factorial design. *R. irregularis* inoculum was procured in the same manner as the previous experiment, and *F. mosseae* inoculum was acquired directly from Plantworks UK Ltd. Kent, and consisted of AMF colonised carrot roots in pumice and zeolite. To create an AMF free treatment comparable to both AMF inoculum substrates the 'no AMF' control inoculum was created by mixing together 1:1 (v:v) *F. mosseae* and *R. irregularis* inoculum, which was then autoclaved twice at (121°C for 10 minutes during 90 minutes run cycle).

The plant growth substrate was prepared by washing Agsorb® 1:5 volume with dH_2O to remove excess solutes, before mixing with silica sand at a ratio of 1:1 (v:v). The subsequent mixture was autoclaved at 121°C and 1.5 L of this sterilised substrate was mixed with 0.5 g slow release fertiliser (Vitax® sterilised bonemeal, Leicestershire, UK) and 50 g of the appropriate AMF inoculum. All treatments received 10 mL of non-fungal filtrates from both inoculums, generated as Hodge (2001).

Barley seeds were surface sterilised as in the previous experiment before sowing directly into the growth substrate to encourage early AMF colonisation at initially seven replicates per treatment. Plants were grown in a glasshouse as previous and watered with deionised water twice weekly. After two weeks growth, plants were fed once weekly for three weeks with 50 mL nutrient solution as in the previous experiment and were fed 100 mL per week thereafter.

Aphids (*S. avenae*), genotype K (originally supplied by Koppert Biological systems, Netherlands) and Co50 (details in Lukasik *et al.* (2013)) were reared on 4 week old barley plants (cultivar Optic), which may have been mycorrhizal, at 15°C in F2 +S Levington compost. To reduce variation caused by differing aphid age and development stages aphids were produced to a standard age by adding adults to barley (cultivar optic) leaves mounted in 2 % agar, 24 hours later the adults were removed and any offspring transferred to two week old barley seedlings (cultivar optic). After 6 weeks of plant growth a one week old aphid (± one day) was added to the base of the stems of the plant where appropriate. All plants were then covered in a muslin mesh for three weeks until harvest.

Harvest, at a total of 8 weeks plant growth, occurred as the first experiment except that any aphids were counted and removed from the plant with a fine paintbrush first. In some cases aphids failed to reproduce and these replicates were not included in subsequent analysis. As such n = 6 for treatments that included the Co50 genotype of *S. avenae* and n = 5 replicates for treatments that contained the K genotype of *S. avenae*, except for the *'F. mosseae,* K aphid genotype' treatment where n = 4. Thus, a third experiment (see section 2.2.3) was set up to test the aphid performance results of the *F. mosseae*

treatments with increased statistical power. The majority of aphids were found on the leaves, so the chemical analysis of the plant tissue was carried out on leaves. Dried leaf material was milled at 24 Hz for 8 minutes and subsequently analysed for C:N using a Costech elemental combustion system 4010 (Costech Analytical Technologies, Inc. California, U.S.A) and P and Si concentrations using a Thermo Scientific™ (Waltham, Massachusetts, U.S.A) portable X-ray fluorescence analyser (Reidinger, Ramsey & Hartley 2012).

2.2.3 The effect of *F. mosseae* colonisation of barley upon *S. avenae* (Experiment 3)

The experimental design was the same as the second experiment, except for the following: no *R. irregularis* treatments were included, and the *F. mosseae* inoculum was added as colonised *Trifolium pratense* L. and *Plantago lanceolata* L. roots in sand:Agsorb® growth medium. AMF free treatments had 10 mL of *F. mosseae* inoculum non-fungal microbial filtrate added.

To attempt to reduce the incidence of the failure of aphid populations to establish, three one week ± one day old aphids were added to plants five weeks after seed sowing. In order to allow for the option of collecting plant material with controlled numbers and location of aphids, the aphids were added to clip cages secured upon the 2nd or 3rd leaf from the top of the main tiller, although plant material was not analysed due to no effect of AMF on aphid performance. Negative aphid controls received an empty clip cage, and all treatments received a further empty clip cage to collect plant material that may have had defences systemically induced by aphid feeding. A muslin mesh was placed around plants of all treatments and after 48 hours both clip cages were opened on each plant and the leaf material inside removed with scissors sterilised with 70 % ethanol. If present, aphids inside the clip cage were added back to the base of the plant stem. The muslin mesh was reapplied until harvest which occurred eight weeks after seed sowing. N= 8, except for '+AMF, K aphid genotype' where n = 7 and '-AMF, Co50 aphid genotype' where n = 6 due to failure of aphids to reproduce.

2.2.4 Statistical analysis

Analysis was carried out using R (R Core Team 2016) version 3.3.2 (2016-10-31) and the packages 'Hmisc', 'lme4', 'lmerTest', 'lsmeans', 'multcomp', 'multcompview' and 'car'. Percentage and proportional data were arcsine transformed before analysis. For the mycorrhizal colonisation screen barley variety was used as an explanatory variable, and for the aphid performance assays AMF and aphid treatments were used as explanatory variables in linear models. For aphid and AMF traits, negative aphid and mycorrhizal controls were not included in the model, respectively. Block was added to models as a

random factor in each case if block addition reduced the AIC value and thus improved the fit of the model. Pearson's correlations were used to test for association between barley variety RLC and root biomass traits. Data were tested for equal variances using a Levene's test. Data used in correlations and the residuals of linear models were tested for normality with a Shapiro-Wilk's test. For correlations data was log or square root transformed to fit a normal distribution and for linear models to fit the model residuals to a normal distribution.

2.3 Results

2.3.1 AMF colonisation of modern barley varieties, traditional varieties and landraces (Experiment 1)

The RLC of barley colonised by *R. irregularis* depended upon the plant variety ($F_{8, 50}$= 12.2, P < 0.001), but there was no trend of this being dependent upon the variety being modern, or a traditional variety/landrace (Figure 3). This was also true of the percentage of arbuscules and vesicles in the roots ($F_{8, 44.2}$ = 3.5, P <0.005; $F_{8, 50}$ = 5.2, P <0.001 respectively, Table 3). *P. lanceolata* grown in separate treatments alongside the barley varieties was colonised 39.3 % ± 0.6, similar to previous levels of colonisation in the growth medium used (Hodge 2001). Fresh and dry root biomass, as well as dry RWR differed between barley varieties tested ($F_{8, 50}$ = 7.3, P <0.001, $F_{8, 38}$ = 6.2, P <0.001, $F_{8, 31.3}$ = 9.6, P <0.001) again, with no trend of this being dependent upon on barley domestication level (Table 3). Across all treatments RLC was negatively correlated with these parameters (root fresh weight; r_{59} = -0.57, P < 0.001, root dry weight; r_{47} = -0.46, P = 0.011 and RWR; r_{46} = -0.47, P =0.001).

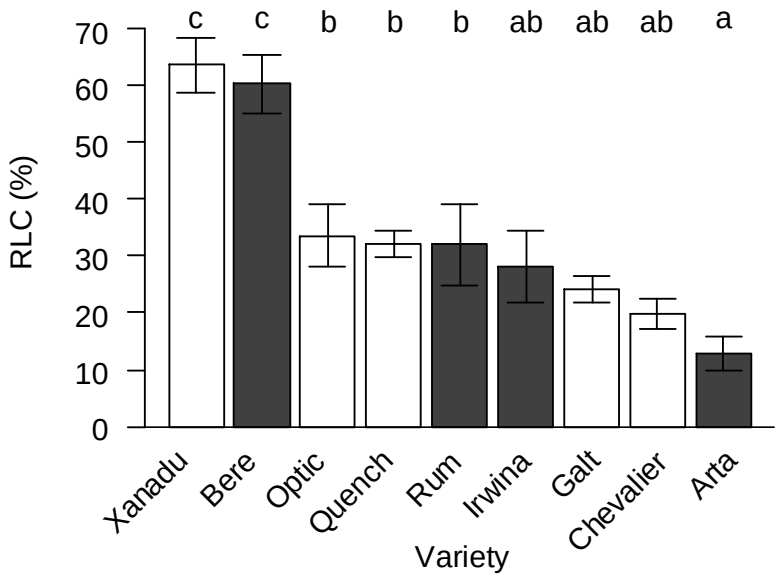

Figure 3. Mean root length colonisation (RLC) of modern barley varieties (open bars), and traditional varieties and landraces (grey bars) colonised by *R. irregularis* at 35 days plant growth. Different letters indicate significant differences at *P* = 0.05 based on a Tukey *post hoc* test. Error bars are ± S.E (n = 7, except for Xanadu, Bere, Irwina and Chev where n =6).

Table 3. Mean (± 1 S.E) root biomass, RWR and root length colonised by vesicles and arbuscules of modern barley varieties, and traditional varieties and landraces.

	Xanadu	Bere	Optic	Quench	Rum	Irwina	Galt	Chev	Arta
Vesicles (%)	9.8 ± 2.8^{bc}	11.2 ± 2.6^{c}	7.2 ± 1.8^{bc}	6.0 ± 1.1^{bc}	3.4 ± 1.4^{abc}	4.3 ± 1.8^{abc}	4.5 ± 1.9^{abc}	3.3 ± 1.8^{ab}	0.4 ± 0.3^{a}
Arbuscules (%)	22.7 ± 4.3^{bc}	23.7 ± 4.7^{c}	12.4 ± 3.5^{abc}	13.7 ± 2.7^{abc}	13.9 ± 3.6^{abc}	10.2 ± 3.2^{abc}	9.8 ± 2.0^{abc}	7.8 ± 1.9^{ab}	6.0 ± 2.0^{a}
Root FW (g)	1.38 ± 0.09^{a}	1.58 ± 0.08^{ab}	2.13 ± 0.18^{abc}	1.47 ± 0.13a	2.09 ± 0.23^{abc}	1.92 ± 0.14^{ab}	2.38 ± 0.22^{bc}	2.01 ± 0.20^{ab}	2.87 ± 0.19^{c}
Root DW (g)	0.23 ± 0.01^{a}	0.35 ± 0.09^{a}	0.60 ± 0.12^{a}	0.30 ± 0.04^{a}	0.52 ± 0.13^{a}	0.57 ± 0.2^{a}	0.82 ± 0.18^{ab}	0.47 ± 0.05^{a}	1.35 ± 0.25^{b}
RWR	0.26 ± 0.02^{a}	0.34 ±0.06^{ab}	0.50 ± 0.07^{bcd}	0.32 ± 0.05^{a}	0.47 ± 0.09^{abc}	0.39 ± 0.1^{abc}	0.55 ± 0.05^{cd}	0.50 ± 0.03^{cd}	0.74 ± 0.06^{d}

2.3.2 Effect of AMF species and aphid genotype in AMF-barley-*S. avenae* interactions (Experiment 2)

No AMF structures were recorded in -AMF treatments, whilst *F. mosseae* colonised the plant to a higher extent than *R. irregularis* (*F. mossae*: 51.8 ± 2.8 % RLC c.f *R. irregularis*: 32.9 ± 1.8 % RLC). Moreover, the roots contained a higher percentage of arbuscules (*F. mosseae*: 26.0 ± 1.9 % arbuscules c.f *R. irregularis*: 13.2 ± 1.8 % arbuscules) and vesicles (*F. mosseae*: 6.6 ± 0.8 % vesicles c.f *R. irregularis*: 2.7 ± 0.9 % vesicles), whilst aphid presence had no effect on any of the AMF characteristics measured (Table 4).

Aphids of the Co50 genotype performed better than that of the K genotype (Table 4; Figure 4), and there was no effect of AMF colonisation by either species on aphid performance.

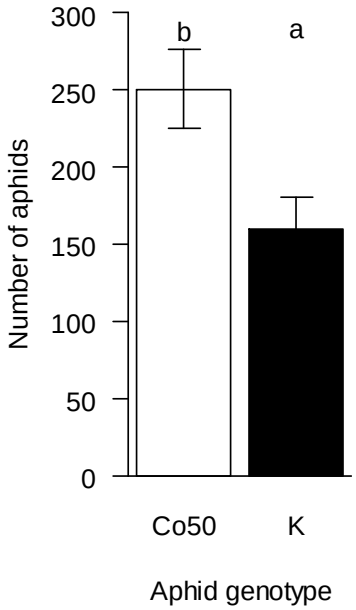

Figure 4. Mean number of aphids on barley plants across all AMF treatments at harvest. Different letters indicate significant differences at *P* = 0.05 based on a Tukey *post hoc* test. Error bars are ± S.E (n = 18 for the Co50 aphid genotype and n = 14 for the K genotype).

Table 4. Results of a general linear model for the effects of aphid treatment (infestation with K genotype, Co50 genotype or no aphid (Aphid)), AMF treatment (colonisation by *F. mosseae, R. irregularis* or no AMF (AMF)) and their interaction on aphid, plant and AMF traits. Where aphid and AMF traits are investigated, treatments without aphids or AMF are removed from the model, respectively.

Response variable	AMF			Aphid			Aphid*AMF		
	D.F.	*F*	*P*	D.F	*F*	*P*	D.F	*F*	*P*
Aphid number	2,26	1.3	0.298	1,26	7.8	**0.010**	2,26	0.9	0.410
Root DW[1]	2,41	6.6	**<0.005**	2,41	0.8	0.459	4,41	0.9	0.473
Stem and grain DW	2,36.5	2.5	0.099	2,36.5	4.9	**0.014**	4,36.4	0.3	0.867
Leaf DW	2,41	0.1	0.886	2,41	1.1	0.254	4,41	0.3	0.846
Total plant DW	2,41	3.6	**0.037**	2,41	2.4	0.104	4,41	0.5	0.762
Leaf [N][2]	2,40	5.2	**0.001**	2,40	0.8	0.456	2,40	1.4	0.257
Leaf [P]	2,41	5.9	**0.006**	2,41	0.6	0.556	4,41	1.8	0.151
Leaf [Si]	2,40	11.0	**<0.001**	2,40	1.0	0.379	2,40	1.0	0.420
RLC[3]	1,26	33.3	**<0.001**	2,26	0.4	0.683	2,26	1.5	0.245
Arbuscules	1,26	21.2	**<0.001**	2,26	0.5	0.621	2,26	0.2	0.793
Vesicles	1,26	14.0	**<0.001**	2,26	0.2	0.824	2,26	1.2	0.321

[1]DW = dry weight (g), [2][ ] =Concentration (mg g^{-1}), [3]RLC, Arbuscules and Vesicles = % of root length.

Below ground plant biomass (measured as root dry weight) was reduced by *R. irregularis* colonisation (-AMF: 0.98 ± 0.1 g c.f *R. irregularis*: 0.56 ± 0.07 g) whilst colonisation by *F. mosseae* resulted in an intermediate effect (0.76 ± 0.09 g; Table 4). Above ground, stem and leaf biomass was reduced by the Co50 aphid genotype (-Aphid:1.74 ± 0.1 g c.f. Co50: 1.35 ± 0.08 g) but plants fed on by the K genotype did not significantly differ from either treatment (1.53 ± 0.11 g; Table 4). Although the majority of the aphids were found feeding on the leaves, neither AMF colonisation nor infestation of the plants with aphids altered leaf biomass (0.92 ± 0.02 g; Table 4). However, colonisation by *R. irregularis* decreased the total plant dry weight compared to –AMF plants (-AMF: 3.60 ± 0.20 g c.f *R. irregularis*: 3.00 ± 0.17) with, similar to root biomass, *F. mosseae* resulting in an intermediate effect (3.07 ± 0.15 g; Table 4).

R. irregularis increased leaf P concentrations above that of –AMF plants (Figure 5a), whilst colonisation of the plant by *F. mosseae* increased leaf N concentrations (Figure 5b). Colonisation by both species of the AMF decreased leaf Si concentrations (Figure 5c); Table 4).

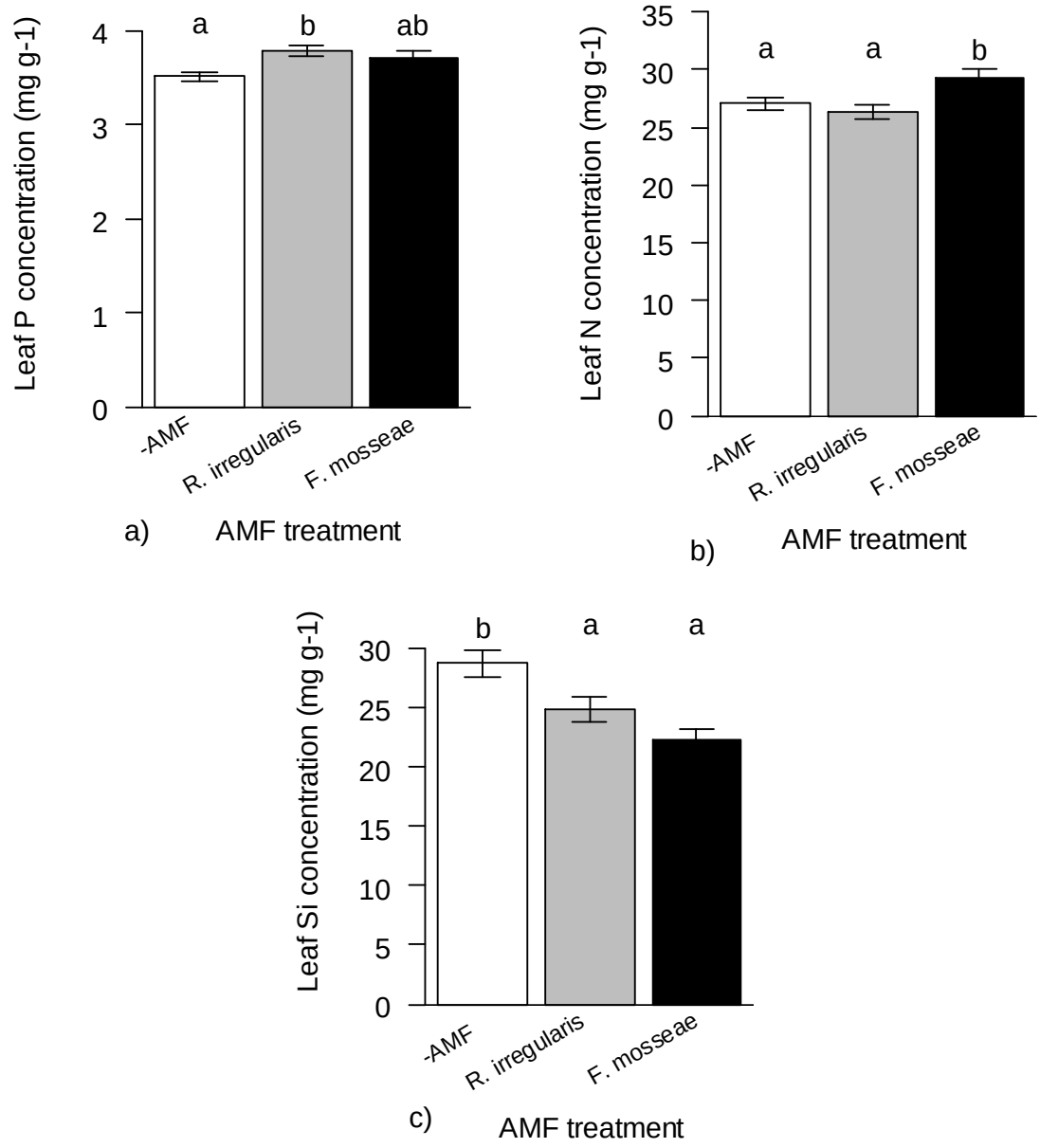

Figure 5. Mean leaf a) P concentration, b) N concentration and c) Si concentration (mg g^{-1}) of barley at harvest of the aphid performance assay. Different letters indicate significant differences at *P* = 0.05 based on a Tukey *post hoc* test. Error bars are ± 1 S.E (n = 17 for – AMF, n = 16 for *R. irregularis* and n = 15 for *F. mosseae*).

2.3.3 The effect of *F. mosseae* colonisation of barley upon *S. avenae* (Experiment 3)

Neither aphid genotype affected the levels of *F. mosseae* RLC (42.5 ± 2.9 % RLC; $F_{2,19}$ = 0.49, *P* = 0.615), arbuscules (22.7 ± 2.2 % arbuscules; $F_{2,19}$ = 0.09, *P* = 0.917) or vesicles (5.6 ± 0.8 % vesicles; $F_{2,12.7}$ = 0.09, *P* = 0.915) in the plant roots at harvest. At harvest, population of the Co50 genotype of *S. avenae* was significantly greater than that of the K genotype (Co50: 510 ± 56 c.f K: 308 ± 64), but this was not affected by colonisation of the plant host by *F. mosseae* (Table 5).

Colonisation by *F. mosseae* reduced root biomass (-AMF: 0.44 ± 0.03 c.f *F. mosseae*: 0.31 ± 0.03). Whilst neither *F. mosseae* nor either of the aphid genotypes affected leaf biomass (0.44 ± 0.02), the Co50 genotype of aphid reduced the stem biomass compared to –Aphid treatments (-Aphid: 0.46 ± 0.03 c.f Co50: 0.30 ± 0.04) and the K genotype induced an intermediate effect (0.40 ± 0.05). This pattern of stem biomass was reflected in the total plant dry weight (-Aphid: 1.38 ± 0.03 c.f K: 1.20 ± 0.05 c.f Co50 0.99 ± 0.04; Table 5).

Table 5. Results of a general linear model for the effects of aphid treatment (infestation with K genotype, Co50 genotype or no aphid (Aphid)), and *F. mosseae* treatment (colonisation by *F. mosseae* or no AMF (AMF)), and their interaction on aphid, number and plant biomass. For aphid number, the -Aphid treatment is removed from the model.

Response variable	*F. mosseae*			Aphid			Aphid*AMF		
	D.F.	*F*	*P*	D.F	*F*	*P*	D.F	*F*	*P*
Aphid number	1,25	<0.1	0.775	1,25	5.1	**0.034**	1,25	0.2	0.697
Root DW[1]	1,39	5.5	**0.024**	2,39	1.9	0.159	2,39	1.0	0.361
Stem and grain DW	1,32.5	0.8	0.383	2,32.4	6.0	**0.006**	2,32.4	<0.1	0.972
Leaf DW	1,39	2.3	0.141	2,39	2.9	0.069	2,39	<0.1	0.922
Total plant DW	1,39	3.4	0.074	2,39	3.4	**0.044**	2,39	0.9	0.147

[1]DW = g

2.4 Discussion

This study aimed to investigate whether modern barley varieties have a reduced capacity to form the AMF symbiosis. Contrary to the hypothesis, both modern and older varieties of barley showed a wide range of colonisation levels by the generalist AMF *R. irregularis*. Thus, a well colonising modern variety of barley, Xanadu, was used in a second experiment to investigate the effect of AMF colonisation of barley upon *S. avenae* performance, and test the impact that AMF species and aphid genotype contexts had on the interaction. Although the effects on plant leaf nutrition were dependent upon the AMF species, neither *F. mosseae* or *R. irregularis* impacted the population growth of aphids, which was dependent upon aphid genotype.

2.4.1 AMF colonisation of modern barley varieties, traditional varieties and landraces (Experiment 1)

We hypothesised that more modern varieties of barley would be reduced in AMF colonisation as these varieties have been bred for high performance under high levels of agrochemical inputs. On the contrary, both modern and older varieties exhibited high or low levels of RLC, suggesting that 'breeding age' does not impact the extent of AMF colonisation in barley roots. This also confirms the results of studies that investigated fewer barley varieties (Zhu, Smith & Smith 2003; Castellanos-Morales *et al.* 2011). The roots of *P. lanceolata* grown in tandem with the study were colonised to an extent expected for the growth medium (Hodge 2001) suggesting good inoculum health. Moreover, the RLC levels of individual barley varieties investigated in Experiment 1, such as 'Xanadu' and 'Galt' reflect levels of RLC reported in other studies (Boyetchko & Tewari 1995; Khaosaad *et al.* 2007; Castellanos-Morales *et al.* 2011).

It is proposed that fungal pathogen resistance may result in lower levels of colonisation (Jacott, Murray & Ridout 2017), but whilst the powdery mildew susceptible landrace 'Bere' (Martin, Chang & Wishart 2010) is readily colonised by AMF in the current study, the highly powdery mildew resistant cultivar 'Xanadu' (Dreiseitl 2015), is similarly colonised. Similarly, fungal pathogen resistance is not related to AMF RLC in maize (An *et al.* 2010) and tomato *Solanum lycopersicum* L. (Steinkellner *et al.* 2012). Whilst single gene mutations such as the 'mlo' genotype may confer reduced susceptibility to both AMF and fungal pathogens in barley (Ruiz-Lozano, Gianinazzi & Gianinazzi-Pearson 1999), the 'Xanadu' variety tested here is also proposed to carry the 'mlo' gene (Dreiseitl 2015) suggesting that the relationship between fungal pathogen resistance and AMF colonisation is complex.

Root architecture may impact plant associations with rhizosphere organisms (Perez-Jaramillo *et al.* 2017). In the current study, the proportion of plant biomass allocated to

roots was negatively associated with RLC. Whilst it is possible that barley varieties with smaller root systems are more reliant upon the AMF symbiosis, further analysis in the second experiment suggests that AMF colonisation may reduce root biomass. Thus, further study is required to disentangle whether the negative association of barley variety root biomass with AMF colonisation drives, or is a response to high levels of AMF colonisation.

For plant breeding efforts that wish to take advantage of microbial symbioses to reduce agro-environmental impacts and increase food security, it is positive that modern varieties of barley can form high levels of AMF colonisation. However, whether levels of RLC amongst barley varieties are associated with a positive growth response requires elucidation. Moreover, the overall growth performance of a crop variety, whether colonised or not by AMF, should also be taken into account when breeding crops to sustain yields with fewer agro-chemical inputs (Sawers, Gutjahr & Paszkowski 2008)

2.4.2 Effect of AMF species and aphid genotype in AMF-barley-*S. avenae* interactions (Experiment 2 and 3)

The second experiment aimed to investigate the effect of AMF colonisation, by two contrasting species, of a modern barley variety on the performance of two genotypes of *S. avenae*. As hypothesised, and reported in other studies (Debarro *et al.* 1995; Zytynska & Preziosi 2011), the performance of *S. avenae* was genotype dependent, perhaps due to the unique elicitation of plant defence genes by different aphid genotypes (Zytynska *et al.* 2016). It was also hypothesised that colonisation of the plant host by *F. mosseae* would reduce aphid performance. However, although *F. mosseae* is more effective at supressing microbial pests of the plant host than *R. irregularis* (Pozo *et al.* 2002; Mustafa *et al.* 2016), neither AMF species affected the number of either aphid genotype at harvest in the second experiment. Similarly, in the third experiment colonisation of the shared host plant by *F. mosseae* did not influence aphid performance but aphid genotype did. This reflects the AMF-*Solanum* spp.-potato aphid (*Macrosiphum euphorbiae* (Thomas)) system (Karley, Emslie-Smith & Bennett 2017), where amongst AMF, plant species and aphid genotype treatment combinations, aphid performance was driven by aphid genotype but not affected by AMF colonisation.

AMF colonisation can increase the performance of *S. avenae* on wheat (Simon *et al.* 2017), but no effect occurred on barley in the current study. How plants interact with aphids can depend on the aphid- plant species combination (Saheed *et al.* 2009; Larsson *et al.* 2011). Thus, it is possible that plant species identity contributes to the different response of *S. avenae* to AMF colonisation of barley and wheat. However, it is also possible that the environmental conditions could be masking the potential effects of AMF upon aphids in the current study. As predicted (Jansa, Smith & Smith 2008; Leigh, Hodge

& Fitter 2009), the plant nutritional status in the second experiment depended upon the AMF species. However, these changes to the nutritional quality of the aphids food source did not impact aphid performance, similar to the AMF-barley-bird cherry oat aphid (*Rhopalosiphum padi* (L.)) system (Williams, Birkhofer & Hedlund 2014). Whilst a decoupling of the impacts of AMF on plant nutrition and pest performance could be promising for food security, it is possible that the alteration to plant nutrition reported in this and the current study were too marginal to influence aphids. It is proposed (Wurst *et al.* 2004) that the restriction of AMF nutrient foraging in single pot systems may mask the potential effects of AMF upon plant nutrition (Thirkell, Cameron & Hodge 2016), and thus aphid performance. To make use of AMF-aphid interactions in more complex agricultural settings to supress aphids, it may be important to consider these contexts. Both species of AMF in Experiment 2 reduced the Si concentration of leaves. As Si is taken up via transporters in the mature roots and root tips of barley (Yamaji *et al.* 2012), this may be due to the reduced root biomass of AMF colonised plants in the current study, though the effectiveness of silicon defence on aphids is unclear (Massey, Ennos & Hartley 2006).

2.5 Conclusions and the selection of a model system for further study

From the varieties investigated in the current experiment, there is no initial evidence that modern breeding practices have reduced the ability of barley to recruit AMF. However, AMF colonisation of a highly mycorrhizal modern barley variety, whether by *R. irregularis* or *F. mosseae*, did not alter aphid performance, even though the effect of the individual species of AMF on host plant nutrient acquisition and growth differed in magnitude. Aphid performance was most dependent on aphid genotype, reinforcing the findings of other studies. An overall aim throughout the experiments outlined in this chapter was to inform the selection of AMF species and barley and aphid genotypes for further study. The modern barley variety ‘Xanadu’ was found to be consistently well colonised by AMF and was selected for further study in experiments using *F. mosseae* and aphids with the K genotype.

3 Aphids can acquire the nitrogen delivered to plants by arbuscular mycorrhizal fungi.

Disclaimer: This chapter (3) was published by Functional Ecology as a research article in January 2019;

‘Wilkinson, T.D.J., Ferrari, J., Hartley, S.E., and Hodge, A. (2019). Aphids can acquire the nitrogen delivered to plants by arbuscular mycorrhizal fungi. *Functional Ecology*. doi:10.1111/1365-2435.13283’.

Notes: This research article has been modified into a chapter format for this thesis and so contains figure, text, table, unit and layout formatting differences, minor spelling and grammatical differences, as well as altered reference styles and altered figure/ table/heading numbers compared to the final published manuscript. The supplementary information is presented in the thesis appendix, and the reference list is combined into a list with the other references found in this thesis. Acknowledgements, conflicts of interest, authors contribution and data accessibility statements found in the final published manuscript are not included in this thesis.

Thomas Wilkinson (me), Sue Hartley, Julia Ferrari and Angela Hodge contributed to the writing of the manuscript. Thomas Wilkinson (me) conducted the experiment and analysed the data.

3.1 Summary

1. Above- and below-ground organisms can interact by altering the quality of shared host plants. Arbuscular mycorrhizal fungi (AMF) influence plant nutrient uptake, including nitrogen (N) acquisition. Under low N and phosphorus conditions, AMF delivery of N from organic sources not immediately available to the plant can have large impacts on plant N status, a limiting nutrient in the aphid diet.

2. This study investigated the effect of AMF colonisation upon aphid number and determined the consequences of AMF directly accessing an organic nutrient patch that the plant cannot. We hypothesised that AMF colonisation of plants will increase plant and aphid N status, plant performance and aphid number, but only when the AMF had direct access to the added organic patch.

3. Barley plants hosting the grain aphid *Sitobion avenae* were colonised by the AMF, *Funneliformis mosseae*, or no AMF. A two-compartment microcosm was used to separate the plant roots from a ^{15}N-labelled organic patch in a second compartment. AMF colonised plants, but without access to the second compartment, were used to examine the effect of AMF colonisation on aphid number. In a separate treatment, and to determine whether AMF access to a plant inaccessible N source modified the effect of AMF colonisation on aphid number, AMF hyphae were permitted access to the second compartment containing an organic patch. As a control for AMF accessing a larger substrate volume, AMF were allowed access to a second compartment without an organic patch.

4. When the AMF accessed the organic patch, more N from the patch was delivered to the plant resulting in a higher grain N concentration although plant growth was depressed. More N from the patch was also delivered to the aphids, but the N status of the aphid remained unchanged. Regardless of the level of access to the organic patch, AMF colonisation did not affect aphid number.

5. Our data show that by accessing N sources not readily available to plants, AMF can indirectly deliver N to above-ground organisms, a finding which has major implications for N-transfer between higher trophic levels.

3.1.1 Key words

^{15}N, arbuscular mycorrhizal fungi, *Funneliformis mosseae*, *Hordeum vulgare*, multitrophic interactions, nutrient acquisition, *Sitobion avenae*, stable isotopic labelling

3.2 Introduction

Via the conduit of a shared plant host, multitrophic interactions can occur among a wide range of below- and above-ground organisms including bacteria, fungi, invertebrates and vertebrates (Gehring & Bennett 2009; Pineda *et al.* 2012; van der Heyde *et al.* 2017a). Due to the diverse organisms that exhibit interactions across the above- and below-ground interface, these interactions have large implications in key ecological processes such as nutrient cycling (Hodge & Fitter 2010; Lau 2011; Grabmaier *et al.* 2014), potent greenhouse gas emissions (Bender, Conen & van der Heijden 2015; Storer *et al.* 2017) and both population and community structure (Gehring & Bennett 2009).

Arbuscular mycorrhizal fungi (AMF) are obligate symbionts that form a mutualistic relationship with the roots of around two thirds of plant species (Hughes *et al.* 2008). AMF benefit their associated plant host in a number of ways including, aiding nutrient acquisition (Harrison, Dewbre & Liu 2002; Leigh, Hodge & Fitter 2009) and resistance to biotic (Koricheva, Gange & Jones 2009; Yang *et al.* 2014; Mauch-Mani *et al.* 2017) and abiotic pressures (Zhang *et al.* 2005; Talaat & Shawky 2012) in return for fixed carbon (C) from the plant. AMF colonisation can also alter the performance of many foliar-feeding herbivores including aphids (Hartley & Gange 2009). Impacts of AMF on aphid performance can be positive (Gange & West 1994; Simon *et al.* 2017), negative (Guerrieri *et al.* 2004; Hempel *et al.* 2009) or have no effect (Williams, Birkhofer & Hedlund 2014; Karley, Emslie-Smith & Bennett 2017). It is currently not fully understood what mechanisms cause such context-specific outcomes, but varying plant-AMF-aphid species, genotypes or environmental conditions may play a role. Although not in direct contact, aphids and AMF could affect the performance of the other via altering the quality of the plant host through modulating defence responses, and/or the plant's phosphorus (P) (Smith & Read 2008) and nitrogen (N) (Thirkell, Cameron & Hodge 2016) status.

N is a limiting factor in an aphid's diet of phloem sap; increased N fertilisation has been shown to increase the performance of *Rhopalosiphum padi* on barley (*Hordeum vulgare* L.) (Ponder *et al.* 2000) and *R. padi* and the English grain aphid (*Sitobion avenae* (F.)) on wheat (*Triticum aestivum*) (Aqueel & Leather 2011). Thus, if AMF altered the plant's N concentration, and hence, the amount of N an aphid had access to per unit time of feeding, this could significantly impact upon aphid performance. However, how increased N fertilisation affects aphid performance may depend on the plant species (Bogaert *et al.* 2017) and aphid morph (Khan & Port 2008). Moreover, the effect of changes to plant host N by AMF upon aphids shows no general trend in the literature (Hempel *et al.* 2009; Babikova *et al.* 2014a; Williams, Birkhofer & Hedlund 2014; Tomczak & Muller 2017), perhaps due to concurrent changes to aphid feeding behaviour, how the N is allocated in the plant tissue or whether it is partitioned into plant defence responses.

The distribution of nutrients in soil is both spatially and temporally heterogeneous. Thus, nutrients do not occur in a uniform manner but as discrete zones or “patches”. Plant root systems have to cope with this heterogeneity in order to acquire the nutrients they need (Hodge 2004), whilst AMF hyphae, by virtue of small size and large surface area, may aid the plant in this nutrient acquisition role through being able to physically access nutrient sources the plant cannot (Hodge & Storer 2015; Hodge 2016). Under poor nutrient conditions, AMF facilitated N-transfer to their associated host plant contributes substantially to the plants N supply, especially when the AMF are able to access an organic nutrient patch that the roots cannot (Leigh, Hodge & Fitter 2009; Thirkell, Cameron & Hodge 2016). Accessing these organic nutrient patches may also affect plant P status (Barrett, Campbell & Hodge 2014; Thirkell, Cameron & Hodge 2016), and although the benefit of P fertilisation on aphid performance is conflicting (Jansson & Ekbom 2002; Venter *et al.* 2014), this could contribute to the growth response of host plants to AMF colonisation.

However, AMF associations with barley in pot systems often induce plant growth depressions (Smith & Smith 2011a). Although growth responses may depend on many plant and AMF partner traits, this is potentially in part due to the fine root structures of cereals resulting in efficient nutrient acquisition. Hence, the AMF symbiosis may not provide a nutritional benefit to the plant that outweighs the C cost of maintaining the symbiosis (Smith & Smith 2011a), which may be further exacerbated in pot systems where the plant roots are able to explore the majority of the soil volume. Therefore, to fully understand whether AMF directly alter aphid performance via nutrient acquisition, we need to determine the effects of AMF access to otherwise plant inaccessible nutrient sources on aphid performance.

In this study, we investigated the effect of AMF colonisation of barley upon plant host biomass and above ground N and P nutrition, and on the number of *S. avenae* sharing the plant host as a surrogate for aphid performance. We investigated whether these plant and aphid characteristics depended upon the AM fungus being able to directly access N contained in organic nutrient patches. Finally, we used stable isotope labelling to assess, for the first time, whether AMF indirectly transfer this N to trophic levels higher than the plant. Specifically the following hypotheses were tested: 1) when the AMF are denied direct access to the organic nutrient patch, AMF colonisation will not enhance nutrient transfer from the patch to the plant and consequently will have no effect on plant N and P status or aphid number. Instead, plant growth will be depressed by the AMF acting as a C drain on the plant for no nutritional benefit. Conversely, 2) when AMF are able to directly access the organic patch, the increased nutrient availability will increase plant biomass, plant N and P status, and aphid number. Finally, 3) when AMF are able to directly access

the organic patch, more N will be transferred, as determined by ^{15}N movement, from this below ground patch to the above ground tissues of the plant and then to its associated aphid herbivores.

3.3 Materials and methods

3.3.1 Experimental design

A two-compartment microcosm design was used to manipulate three aspects of the plant-AMF symbiosis: a) whether the plant was colonised by AMF (+AM) or not (-AM), b) the impact of access to increased physical volume on the nutrient foraging availability of the AMF (+Access/ -Access) and c) whether this access led to direct AMF access to an organic nutrient patch (+Patch/-Patch) (Figure 6). Due to the nature of the hypotheses being tested the design was not fully factorial (Table 6).

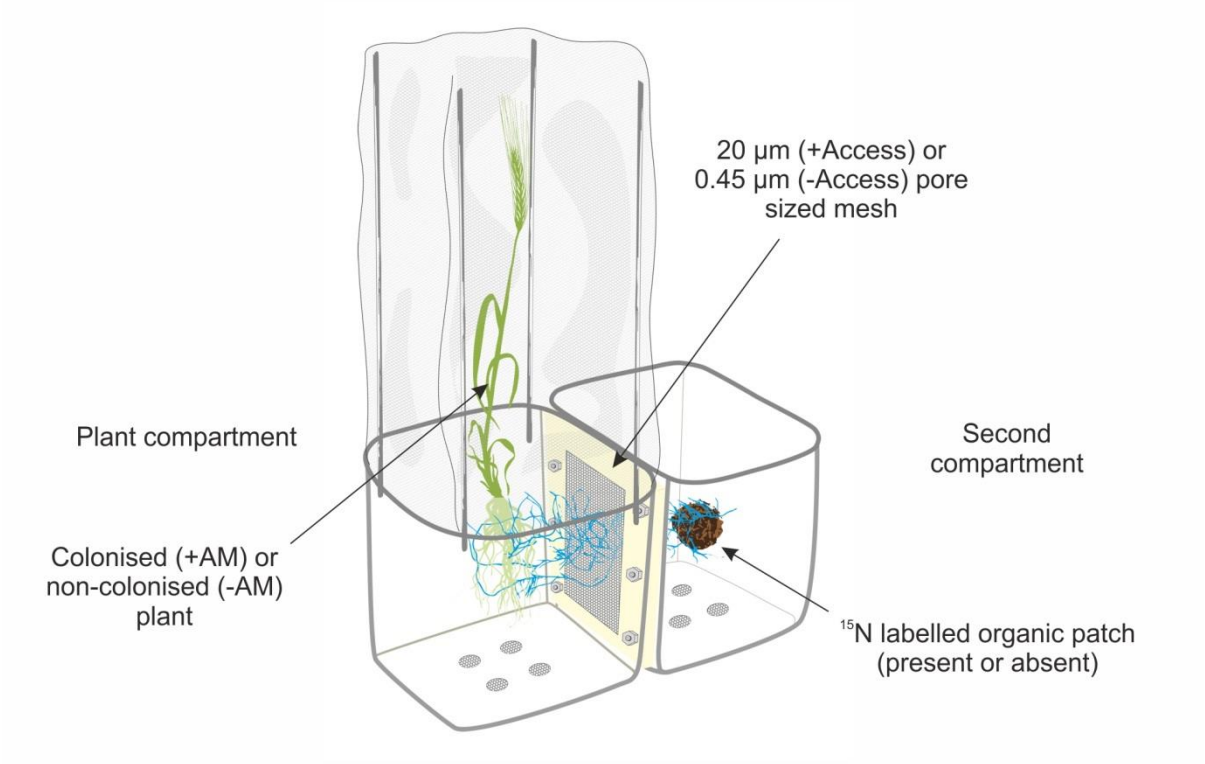

Figure 6. Illustration of the two compartment experimental microcosm unit. Plant roots are confined to the plant compartment only, while arbuscular mycorrhizal fungi (AMF) hyphae (blue lines) are permitted (20 µm mesh) or denied (0.45 µm mesh) access to the second compartment. The second compartment contained a ^{15}N-labelled organic nutrient patch of milled barley shoot material except in the “volume control” treatment which was included to examine the impact of AMF access to increased physical space on the AMF-plant-aphid interaction. The system investigates the effect of direct access of AMF to nutrients by allowing the AMF, if present, access to the second compartment containing an organic patch.

Table 6. AMF colonisation, AMF access to the second compartment and patch presence treatment combinations.

Treatment name	Treatment design			Purpose of treatment	Hypothesised effect compared to "-AM"			
	AMF colonisation	Potential AMF access to second compartment	Organic patch presence		Plant bio-mass	Plant N and P	N delivery from the patch above ground	Aphid number
-AM	None	+Access	+Patch	Control for hypotheses 1), 2) and 3). As plant roots are always restricted to the plant compartment by the use of meshes, a "-AM, -Access, -Patch" treatment is redundant.	NA	NA	NA	NA
+AM, -Access	Yes	-Access,	+Patch	Tests hypothesis 1) and 3). Compare with (-AM) to test the effect of AMF colonisation when AMF cannot directly access more nutrient sources than the plant.	↓	=	=	=
+AM, +Access	Yes	+Access,	+Patch	Tests hypothesis 2) and 3). Compare with (-AM) to test effect of AMF colonisation when the AMF can directly access nutrient sources the plant cannot.	↑	↑	↑	↑
Volume control	Yes	+Access,	-Patch	"Volume control" for hypothesis 2). Compare with (+AM, -Access) to test for the effect of AMF exploring more physical volume (growth substrate). If the volume of growth substrate has no effect, plant and aphid nutrient responses will not differ from "+AM, -Access". If volume does have an effect, then comparison with "+AM, +Access" enables the effects of the nutrient patch to be studied.	↓	=	NA	=

Notes. Arrow direction represents the hypothesised effect of the treatment compared to the non-colonised plant. '=' represents no effect, 'NA' represents no hypothesis in comparison to the "-AM" treatment.

Comparison of non-AMF plants (-AM) with AMF plants where both roots and AMF access were confined to the plant compartment (+AM, -Access) allowed hypothesis 1 to be tested. As the “+Access” treatment only refers to the access by the AMF hyphae and not plant roots, and neither “Access” treatment impedes the movement of small solutes, an “Access” treatment with respect to the “-AM” treatment is redundant. Hypothesis 2 was investigated by comparing non-colonised plants (-AM) with plants colonised by AMF allowed to directly access the organic patch in the second compartment (+AM, +Access). To minimise differences in nutrient availability in the plant compartment due to the mass flow or diffusion of solutes from the organic patch, an organic patch was added to the “-AM” and “+AM, -Access” as well as the “+AM, +Access” treatment. As the effect of AMF access to, rather than the presence of an organic nutrient patch was investigated, “-Patch” treatments were not combined with “-AM”, and “+AM, -Access” treatments. However, when the AMF has access to the second compartment (+AM, +Access) it also has access to a larger volume of growth substrate. Thus, a “volume control” was generated by allowing AMF access to the second compartment, but with no organic nutrient patch present.

3.3.2 Microcosm design

Microcosms were adapted from the design by Hodge and Fitter (2010), and comprised of two identically sized polypropylene compartments each measuring 14 x 14 x 13 cm. A 6 x 4 cm window was cut into the adjoining sides of the compartments and covered with a double layer of either 20 µm (John Stanier and Co., Whitefield, Manchester, UK) or 0.45 µm (Santa Cruz Biotechnology, Dallas, Texas, US) nylon mesh used to control the access of AMF hyphae to the second compartment. The 20 µm mesh permits AMF to penetrate from the plant compartment into the second compartment (used in AMF “+Access”) treatments whereas the 0.45 µm does not (used in AMF “-Access” treatments).

3.3.3 AMF inoculation and plant growth

Silica sand was mixed 1:1 (v/v) with Agsorb® (a calcinated, attapulgite clay particle soil conditioner (Oil-Dri, Cambridgeshire, UK)) washed twice with deionised water and inoculated with AMF to produce the growth substrate added to the plant compartment of AMF treatments (+AM).

The AMF inoculum was *Funneliformis mosseae* (Plantworks UK Ltd. Kent, UK), added as colonised roots of *Plantago lanceolata* L. and *Trifolium pratense* L. in a sand: Agsorb® medium (1:1 (v/v)). Bonemeal (0.25 g L^{-1}; Vitax Leicestershire, UK) was also added as a complex N and P source to encourage AMF development (Hodge & Fitter 2010).

The growth substrate for the plant compartments of non-AMF treatments (-AM) were set up in the same manner except the AMF inoculum was autoclaved twice at 121°C. In an attempt to equalise starting non-fungal microbial communities between +AM and -AM treatments, prior to autoclaving the AMF inoculum was washed with dH_2O, filtered through 20 µm nylon mesh to remove large particles, then subsequently filtered through Whatman No.1 filter paper (pore size 11 µm, Whatman plc, Maidstone, Kent, UK) and 10 mL of the resulting filtrate added to -AM treatment plant compartments as Hodge (2001).

Sand and Agsorb® was washed and mixed as previously described and added to the second, unplanted, compartment except no AMF inoculum, filtrate or bonemeal was added. Aluminium foil was used to cover the surface of the second compartment to reduce evaporation and algal growth.

Three barley seeds (cultivar: Xanadu, SAATEN-UNION GmbH, Isernhagen, Hanover, Germany) were surface sterilised in 4 % (w/v) sodium hypochlorite solution before being germinated in the centre of the plant compartment. Seedlings were thinned to one per pot after one week and fed weekly with 50 mL of a low N and low P nutrient solution (see Leigh, Hodge and Fitter (2009)) to produce a low N and P system where the effects of N acquisition from an organic patch via AMF may be substantial (Thirkell, Cameron & Hodge 2016). Microcosms were arranged in a randomised block design in a lit and heated glass house with a 16 hour day length on the 27/04/16. Plants were treated and harvested in block order and watered with up to 100 mL dH_2O twice weekly as required.

3.3.4 ^{15}N-labelled organic patch material generation and addition

Barley seedlings (cultivar: Optic, Syngenta UK Limited, Cambridgeshire, UK) were labelled with ^{15}N to produce labelled organic material for use as the organic 'patch'. Briefly, barley seedlings were grown in sand and Agsorb® medium and fed with nutrient solution (Thornton & Bausenwein 2000) containing 1:1 $^{14}NH_4{}^{14}NO_3$: 98 atom % ^{15}N $^{14}NH_4{}^{15}NO_3$ (Sigma Aldrich, St. Louis, Missouri, United States). The resulting labelled shoot material was dried at 70°C, homogenised in a ball mill (Retsch MM400, Retsch GmbH, Haan, Germany) and analysed by Isotope Ratio Mass Spectrometry (I.R.M.S) (PDZ 2020, Sercon Ltd, Crewe, UK). The shoot material contained 0.52 % P, 2.1 % N of which 4 % was ^{15}N.

A 7-cm-deep, 1-cm-diameter core of growth substrate, 2 cm away from the centre of the mesh window was removed from the second compartment two weeks after the seedlings were thinned to one seedling per pot. For "+Patch" treatments 2.2 g of the ^{15}N-labelled milled shoot material was added into the resulting space, as a discrete layer or 'patch' and the cored substrate replaced on top of the patch material. The organic material added contained 45.98 mg N, of which 1.82 mg was ^{15}N. The "-Patch" treatments were treated in

the same manner, although no organic material was added and the removed substrate was simply replaced.

3.3.5 Aphid rearing and treatment

After 8 weeks growth, one week previous to aphid addition, the outline of all experimental plant live leaves were traced on 100 g m^{-2} paper and subsequently weighed to give a non-destructive estimate of leaf area. Adults of a single genotype of *S. avenae* (genotype; K, originally supplied by Koppert Biological Systems, The Netherlands) raised on barley (cultivar: Optic) at 20°C were transferred to 2-week-old seedlings with roots fixed in 2 % agar in water in a Petri dish, and allowed to birth for 48 hours before being removed. The resulting offspring were allowed to grow on the seedlings for 6 more days. Three of these 7 day ± 1 day aphids were transferred to the base of the stem of the main tiller of each experimental plant and a muslin mesh supported by bamboo canes was placed over all plants.

3.3.6 Harvest and analysis of plant, aphid and AMF traits

Two weeks after aphid addition and 11 weeks after planting, the aphids were removed from plants with a fine paintbrush and the number found on the stem and leaf fraction, or on the grain fraction recorded, before flash freezing and storage at -80°C. A 1-cm-diameter core was taken at the site of the 'patch' to determine the hyphal length density (HLD) in this zone. Plants were separated into root, stem, leaf and grain fractions. Subfractions of roots were stored in 40 % ethanol for AMF staining. The remaining plant material was oven dried at 70°C for at least 72 hours.

Aphids were lyophilised for 24 hours using a Lyotrap (LTE Scientific Ltd, Oldham, UK) and analysed via I.R.M.S for bulk and ^{15}N using a Finnigan DELTA plus XP (ThermoFischer Scientific, Waltham, Massachusetts, USA). Plant material was milled to a fine powder before being pelleted for X-ray fluorescence (XRF) to assess P concentration using a Thermo Scientific ^{TM} portable X-ray fluorescence analyser (as described in Reidinger, Ramsey and Hartley (2012)) and also for bulk N and ^{15}N analysis via I.R.M.S. Growth substrate cores were processed for HLD according to Staddon, Fitter and Graves (1999), and HLD estimated using the gridline intercept method (Hodge 2003). However, the dried growth substrate was lost and so the HLD was calculated per fresh weight rather than dry weight. Roots were stained for AMF colonisation assessment using the acetic acid-ink staining method (Vierheilig *et al.* 1998) but with the following modifications: roots were rinsed with dH_2O then incubated in 10 % KOH at 70°C for 45 min or until the roots became translucent. Roots were thoroughly rinsed with dH_2O again, before being stained with 5 % acetic acid, 5 % Pelican 4001 Brilliant Black ink® (Pelikan International, Feusisberg, Switzerland) and 90 % dH_2O for 30 minutes. Root length colonisation (RLC)

was assessed using the line intercept method under 200 x magnification using a Nikon eclipse 50i upright microscope (Nikon UK Ltd, Surrey, UK) and a minimum of 100 intercepts (Hodge 2003).

3.3.7 Statistical analysis

Analysis was carried out using R version 3.3.2 (2016-10-31) (R Core Team 2016), and the packages, "lme4", “lmerTest”, “lsmeans”, “car”, “userfriendlyscience, “multcomp” and “multcompView”. A Levene’s test was used to test data for equal variances and where this assumption was correct, data were analysed using linear models with treatment (as detailed in Table 6) as the explanatory variable. Block was added as a random factor if the model containing block had a significantly lower AIC value. A Shapiro-Wilk test was used to test for the normal distribution of model residuals and when these were not normally distributed, percentage/proportion data were logit or square root transformed and concentration data log transformed, which allowed the assumptions of the linear model to be met. A Tukey *post hoc* test was used where appropriate. A Welch’s ANOVA with a Games Howell *post hoc* test was used to analyse data with unequal variances. For the analysis of AMF traits the “-AM” data was not included, and for ^{15}N data “volume control” treatment data was not included in the model as data followed a baseline and were thus distributed similar to zero values. Initially plants were set up with 10 replicates per treatment although for one replicate of the “volume control” treatment aphids did not reproduce and this replicate was removed from the subsequent analysis.

3.4 Results

3.4.1 AMF characteristics

Plant roots of +AM treatments were well colonised (*c*. 49 ± 2.9 S.E % RLC; 13 ± 1.6 % vesicles and 15 ± 1.6 % arbuscules; see Figure 19 in Appendix), whilst no AMF colonisation was found in the “-AM” treatment. Neither access to, or the presence of, an organic patch in the second compartment influenced RLC, or the percentage of vesicles and arbuscules ($F_{2,26}$ = 0.16 , P = 0.82; $F_{2,26}$ = 0.24, P = 0.79; $F_{2,26}$ = 0.62, P = 0.55 respectively). However, AMF access to the second compartment, and the presence of the organic patch influenced HLD in the ‘patch zone’ ($F_{2,25}$ = 6.4, P < 0.01). When the AMF had access to the second compartment with an organic patch (+AM, +Access), the HLD values were significantly greater (0.41 ± 0.07 m hyphae g^{-1} fresh substrate) than when no AMF access was permitted (+AM, -Access: 0.13 ± 0.06 m hyphae g^{-1} fresh substrate), whilst HLD in the treatment with AMF access to the second compartment without an organic patch (volume control) did not significantly differ from either of these treatments (0.29 ± 0.08 m hyphae g^{-1} fresh substrate).

3.4.2 Plant characteristics

To investigate the effects of AMF colonisation on plant biomass and above-ground nutrient status when the AMF cannot directly access the organic nutrient patch, a treatment where the AMF was restricted to the plant compartment (+AM, -Access) was included in the experimental design. Compared to non-AMF plants (-AM), this treatment (+AM, -Access) resulted in a reduction of live plant leaf area one week prior to aphid addition (38.9 ± 5.7 cm^2 c.f. 53.9 ± 3.9 cm^2; $F_{3,26.1} = 4.7$, $P < 0.01$). In contrast, the other +AMF colonisation treatments did not differ from the non-AMF plants. The +AM, -Access plants also showed reduced total dry weight at harvest compared to non-AMF plants, but increased grain, stem and leaf P concentrations (Table 7).

Table 7. Plant biomass (dry weight; dw) and above-ground tissue nutrient responses (mean ± 1 standard error) to AMF colonisation, AMF access to the second compartment and organic patch presence at harvest.

	AM, access and organic patch presence or absence				Test statistics		
	-AM	+AM, -Access	+AM, +Access	Volume Control	D.F.	*F*	*P*
Root dw (g)	0.35 ± 0.04	0.23 ± 0.02	0.27 ± 0.04	0.28 ± 0.04	3,35	1.92	0.145
Stem and leaf dw (g)	1.20 ± 0.16^{b}	0.65 ± 0.06^{a}	0.79 ± 0.09^{a}	0.94 ± 0.07^{ab}	3,35	5.12	**0.005**
Grain dw (g)	1.43 ± 0.15^{cb}	0.91 ± 0.07^{a}	0.95 ± 0.03^{b}	1.34 ± 0.08^{c}	3,17	8.45	**0.001**
Total plant dw (g)	2.98 ± 0.33^{b}	1.80 ± 0.15^{a}	2.01 ± 0.13^{ab}	2.56 ± 0.17^{b}	3,19	5.67	**0.006**
Stem and leaf P concentration (mg g^{-1})	2.09 ± 0.13^{a}	2.69 ± 0.19^{b}	3.21 ± 0.18^{b}	2.80 ± 0.17^{b}	3,33	8.45	**<0.001**
Grain P concentration (mg g^{-1})	3.09 ± 0.12^{a}	3.61 ± 0.14^{b}	3.77 ± 0.10^{b}	3.52 ± 0.09^{ab}	3,34	6.18	**0.002**
Stem and leaf N concentration (mg g^{-1})	2.55 ± 0.30	2.92 ± 0.53	3.76 ± 0.49	3.61 ± 0.42	3,35	2.23	0.103

Notes. Different letters within rows indicate significant differences at P = 0.05 using a Tukey, or a Games Howell post hoc test and are shown in bold. D.F = degrees of freedom.

However, neither the grain N concentration, or the amount of grain N originating from the organic patch, of the +AM, -Access plants differed significantly from that of the non-AMF plants (Figure 7; Table 8), but the *proportion* of grain N derived from the patch for these plants was lower than any other treatment (Figure 8a). No treatment altered the N concentration of the stem and leaf (Table 7), the stem and leaf N:P ratio (1.16 ± 0.05), or the grain N:P ratio (3.01 ± 0.05) ($F_{3,32}$ = 1.1, P = 0.37, and $F_{3, 33}$ = 1.1, P = 0.37, respectively).

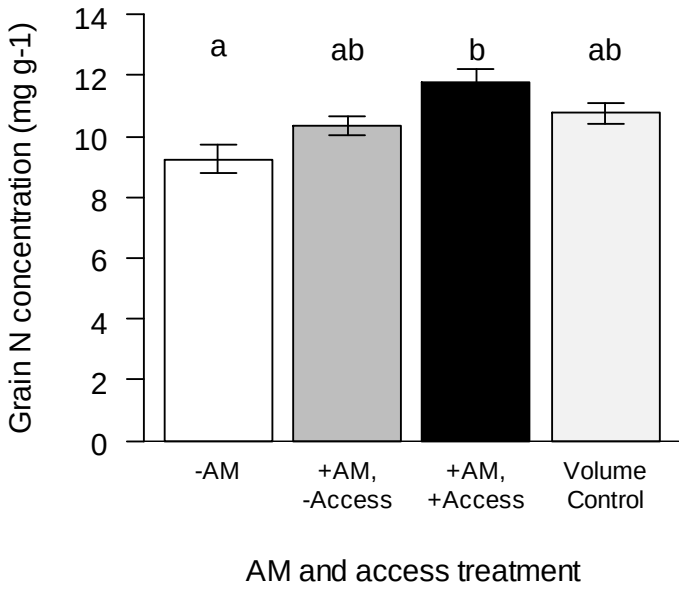

Figure 7. Mean grain N concentration of AMF colonisation and AMF access treatments at harvest. Different letters indicate significant differences at P = 0.05 based on a Tukey *post hoc* test. Error bars are ± standard error of the mean (n = 10, except for "volume control" treatments where n = 9). AMF colonisation and access to the organic nutrient patch in the second compartment significantly increased the grain N concentration ($F_{3,35}$ = 7.2, P < 0.001).

Table 8. Above-ground plant and aphid ^{15}N tissue responses (mean ± 1 standard error) to AMF colonisation and AMF access to the second compartment containing an organic patch at harvest. Note aphid ^{15}N enrichment is expressed as ng whilst other ^{15}N enrichments are expressed as µg.

	AM colonisation and access			Test statistics		
	-AM	+AM, -Access	+AM, +Access,	D.F.	*F*	*P*
Total shoot ^{15} N enrichment (µg)	16.23 ± 2.27^{b}	5.73 ± 2.02^{a}	43.83 ± 7.24^{c}	2,27	22.05	**<0.001**
Stem and leaf ^{15} N enrichment (µg)	1.87 ± 0.30^{b}	0.71 ± 0.25^{a}	6.77 ± 3.05^{b}	2,27	13.90	**<0.001**
Stem and leaf N derived from patch (%)	1.88 ± 0.35^{ab}	0.89 ± 0.30^{a}	4.12 ± 0.73^{b}	2,27	11.32	**<0.001**
Grain ^{15} N enrichment (µg)	14.36 ± 2.15^{a}	5.02 ± 1.97^{a}	37.06 ± 5.28^{b}	2,27	22.36	**<0.001**
Aphid ^{15} N enrichment (ng)	0.50 ± 0.12^{a}	0.45 ± 0.18^{a}	1.36 ± 0.28^{b}	2,18	11.68	**<0.001**

Notes. Different letters within rows indicate significant differences at a level of $P = 0.05$ using a Tukey post hoc test and are in bold. DF = degrees of freedom.

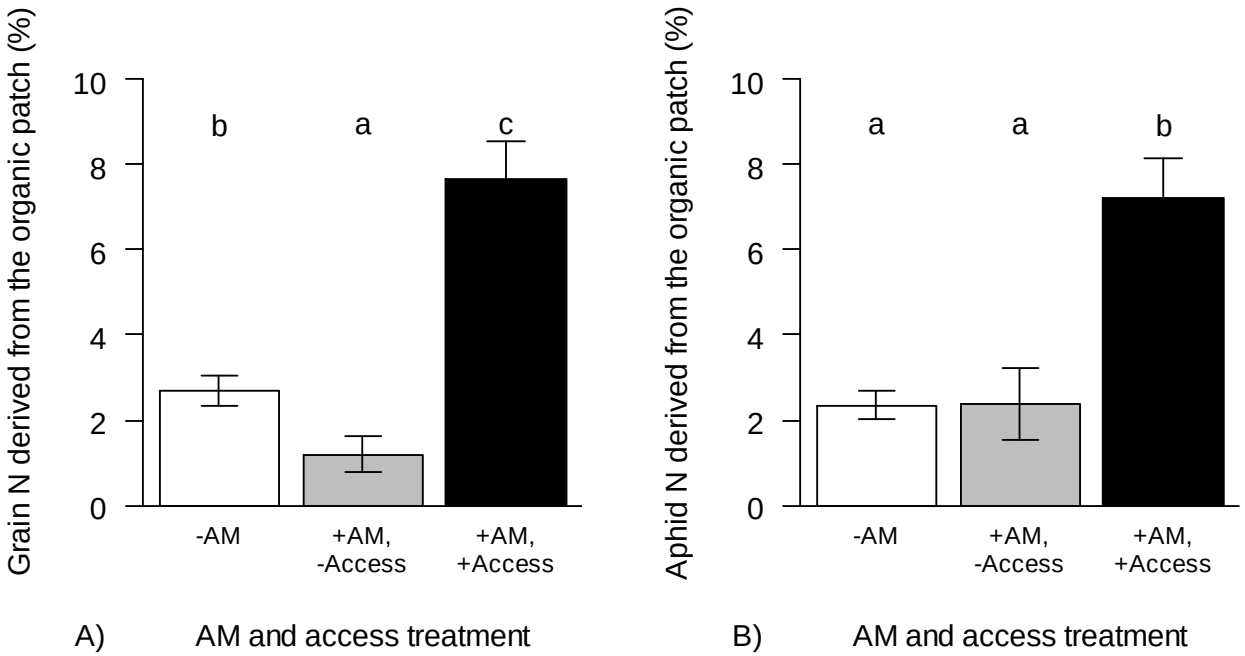

Figure 8. Mean percentage (%) of nitrogen (N) in the A) grain and B) aphid tissues derived from the added organic patch of AMF colonisation and AMF access treatments at harvest. Different letters indicate significant differences at $P = 0.05$ based on a Tukey *post hoc* test. Error bars are ± standard error of the mean (n = 10). AMF colonisation with access to the organic nutrient patch in the second compartment significantly increased the proportion of N derived from the organic nutrient patch contributing to the grain and the aphid ($F_{2,27}$ =21.7, $P < 0.001$; $F_{2,27}$ = 8.7, $P < 0.01$; respectively).

To investigate the effect of AMF colonisation when the AMF can directly access more nutrients than the plant hosts roots, the AMF were allowed to access a second compartment containing an organic nutrient patch (+AM, +Access). This did not alter total plant biomass compared to non-AMF (-AM) plants, but increased above ground P concentrations (Table 7). Moreover, the grain N concentration was increased (*c*. 28 %) compared to non-AMF plants (Figure 7), and more grain N was derived from the organic patch (Table 8). Consequently, the proportion of grain N that originated from the organic patch was highest in this treatment (7.8 % ± 0.93; Figure 8a).

A volume control was included and allowed AMF access to the second compartment but without an organic nutrient patch present. This did not alter grain N or P concentrations compared to the other treatments, but, unlike when the AMF was completely denied access to the second compartment (+AM, -Access), did not induce a reduction of grain biomass compared to non-AMF (-AM) plants (Table 7). Moreover, the grain biomass of the volume control was higher than the other AMF colonised treatments, although the mean grain biomass was highest when plants were not colonised by AMF (-AM). However, a Games Howell *post hoc* test did not identify this as being significantly different from that of the "+AM, +Access" treatment (*P = 0.051*) (Table 7).

3.4.3 Aphid Characteristics

The majority of the aphids were found feeding upon the grain (80 % ± 0.03 S.E), and the mean number of aphids feeding on each plant (51.8 ± 2.7, $F_{3,35}$ = 0.09, *P* = 0.97), total aphid biomass (7.1 ± 0.5 mg, $F_{3,35}$ = 0.24, *P* =0.87), aphid N concentration (62.3 ± 1 mg g^{-1}, $F_{3,35}$ = 1.1, *P* = 0.38) and the total N content in the aphid population (0.44 ± 0.03 mg, $F_{3.35}$ = 0.005, *P* = 0.99) did not differ amongst any treatments. However, allowing the AMF to access the organic patch material (+AM, +Access) resulted in more N being delivered to the aphid population (Table 8) and more of the N in the aphid being derived from the organic patch (7.21 % ± 0.95 S.E., Figure 8b).

3.5 Discussion

This study used a two-compartment microcosm design to investigate the direct effect of AMF access to a complex N source on barley and its associated aphid herbivores under low N and P conditions. We reconfirmed that by directly accessing nutrient patches that the plant cannot access, AMF may deliver previously unavailable N from such patches to the host plant (see Barrett, Campbell and Hodge (2014); Thirkell, Cameron and Hodge (2016)). We also demonstrate, for the first time, that this may also be true for herbivores sharing the host plant. It was hypothesised that the delivery of this N would lead to an increase in plant performance and aphid number. This was reflected in some aspects of the plant's performance, but interestingly, not in the aphids feeding upon it.

3.5.1 Effects of direct AMF access to organic nutrient patches on plant growth and nutrition

We hypothesised that when the AMF were denied access to the second compartment containing an organic patch, the cost of the AMF symbiosis to the plant would outweigh its nutritional benefits. Supporting this hypothesis, plants colonised by AMF restricted to the plant compartment derived the least amount of N from the organic patch, did not increase above ground N concentrations, and displayed the most severe growth depression compared to non-AMF plants (Table 7; Table 8). Whilst the above ground P concentrations were higher in these "+AM, -Access" plants than in non-AMF plants, this could be due to reduced plant biomass. Conversely, we hypothesised that increased nutrient transfer to the host plant would occur when the AM fungus could directly access the organic patch.

We cannot disentangle whether the increased above-ground P concentrations in plants colonised by AMF accessing the organic patch is due to reduced biomass or increased P transfer from the patch, but as expected, these plants were the most highly enriched with the ^{15}N used to label the patch material. This suggests that the increase in grain N concentration above that of the non-AMF plants was not due to reduced plant biomass, and as the live leaf area of these treatments did not differ, it is likely that differences in ^{15}N enrichment are due to AMF-mediated delivery and not driven by mass flow.

Contrary to expectations, this AMF-mediated delivery of nutrients from the organic patch did not positively impact plant biomass. Moreover, the grain biomass was reduced in comparison to when the AMF were allowed to access the second compartment but without a nutrient patch present. AMF may become more beneficial to the plant host with increasing pot size (Audet & Charest 2010; Zangaro *et al.* 2015), and the current study provides evidence that this is due to the AMF being able to explore growth substrate beyond the reach of the plant roots. However, AMF have relatively high N demands and

can act as a sink for patch derived N (Hodge & Fitter 2010; Herman *et al.* 2012), hence, under low N conditions, as used in this study, AMF may become less mutualistic (Johnson *et al.* 2010; Puschel *et al.* 2016), perhaps due to AMF fulfilling their own nutritional requirements first, before passing nutrients to the host. Thus, it is possible, given that AMF proliferate their hyphae extensively upon organic patch interception (Ravnskov *et al.* 1999; Hodge 2001), that this may have drawn more carbon from the plant host without an equal return in N, resulting in the growth depression found when the AMF had access to the organic patch present in the second compartment.

This plant biomass reduction contrasts to previously reported effects in a low N and P context (Thirkell, Cameron & Hodge 2016). Evidence suggests that AMF rely upon the plant host for the energetically expensive process of fatty acid synthesis (Keymer & Gutjahr 2018), although nutritional and plant–AMF partner genetic contexts also play roles in whether plant growth depression occurs (Jin *et al.* 2017). This imbalance may have been exacerbated by above-ground herbivory leading to increased C allocation to the roots (Wamberg, Christensen & Jakobsen 2003).

3.5.2 The effect of direct AMF access to nutrients on aphid number and N status

We hypothesised that the nutritional benefit of AMF directly accessing an organic patch would be passed on to the plant's associated herbivores. The amount of N derived from the patch in the aphid closely relates to that of the grain (i.e. 7.2 %; aphid v. 7.8 %; grain), which reflects where the majority of aphids were found. This suggests that the AMF transfer of N from the patch to the grain, leading to increased grain N concentration, made more N available to the aphid per unit time. This change in concentration (*c.* 28 % between non-AMF plants and AMF plants accessing the organic patch) is similar in magnitude to changes in leaf N concentrations of *Miscanthus* species in which altering N regimes impacted aphid performance (Bogaert *et al.* 2017). However, in the present study, aphid number and N status remained unchanged, suggesting that the aphids did not make use of the extra N made available to them by the AMF, and that aphid and plant N status can be uncoupled. One caveat of our experimental design is that we measured aphid numbers rather than more detailed components of aphid performance (such as survival or individual fecundity), which may might reveal more subtle effects of increased N concentration.

During their life cycle, aphids can compensate for initial reductions in their biomass that are caused by AMF induced changes to plant nutrition (Tomczak & Muller 2017). The ratio of essential to non-essential amino acids in the phloem is a driver of the host plants nutritional quality to aphids (Douglas 2006), with the symbiotic bacteria *Buchnera aphidicola* synthesising essential amino acids lacking from this diet (Guenduez & Douglas

2009). Thus, the composition of essential amino acids in the phloem, the synthesis of amino acids by *B. aphidicola,* and the aphid's ability to alter its length of feeding (Ponder *et al.* 2000), could all influence any effects of altered host plant tissue nutrient concentrations on aphid performance. Although we were not able to measure amino acid availability to the aphid (Johnson, Ryalls & Karley 2014), by tracking and measuring the N status of the aphid alongside that of their host plant, we show that, at least in certain cases, aphids can adjust their overall N intake when the nutritional quality of a plant host is altered by AMF colonisation. This may aid in explaining the contrasting effects reported of AMF-induced changes to plant host N nutrition upon aphid performance (Hempel *et al.* 2009; Babikova *et al.* 2014a; Williams, Birkhofer & Hedlund 2014), alongside potential simultaneous synergistic or confounding effects of AMF-induced changes to plant defence (Mauch-Mani *et al.* 2017). For example, a significant defence for barley, as for many grasses, is silicon (Hartley & DeGabriel 2016) and levels of foliar silicon have been shown to be increased by the presence of AMF (Anda, Opfergelt & Declerck 2016). Silicon uptake is likely to be relatively unaffected by any changes in plant growth-defence trade-offs resulting from colonisation (Simpson *et al.* 2017), though reductions in levels of other barley defences in relation to nutrient content and growth have been observed (Norbaek *et al.* 2003).

Although the nutritional status of the growth medium in our microcosm was low compared to a conventionally managed agriculture systems, our results demonstrate that via the shared host plant, AMF can transfer N derived from complex N sources not directly available to the plant to higher trophic levels. This supports our hypothesis that direct AMF access to organic nutrient patches will result in more N being transferred to above-ground organisms and highlights the importance of AMF in below- and above-ground N cycling.

4 The effects of aphid herbivory upon fungal and AMF communities and characteristics in an agricultural system

4.1 Introduction

Arbuscular mycorrhizal fungi (AMF), sub-phylum Glomeromycotina, form obligate symbioses with the roots of most plant species, including agriculturally important cereals (Smith & Read 2008). Enhancing, and thus, understanding, this symbiosis has been proposed as an important tool in both simultaneously increasing food security and agricultural sustainability (Jacott, Murray & Ridout 2017; Thirkell *et al.* 2017). Whilst the plant host provides a fixed carbon (C) source for AMF, AMF transfer nutrients such as nitrogen (N) and phosphorus (P) to the plant host (Smith & Read 2008). Moreover, AMF colonisation may enhance the uptake of nutrients such as silicon (Si) that alleviate plant stresses (Dias *et al.* 2014; Garg & Bhandari 2016; Frew *et al.* 2017a), as well as drive multitrophic interactions with the above- and below-ground pests of their associated host plant (Yang *et al.* 2014). These interactions can protect the plant from pests and hence, could potentially reduce pesticide use (Thirkell *et al.* 2017). This is of increased importance in the context of emerging pesticide resistant populations such as in the phloem feeding cereal pest, the English grain aphid (*Sitobion avenae* (F.)) (Foster *et al.* 2014).

The bottom up effect of AMF colonisation of the shared host plant upon aphid performance can range from positive to negative (Gange & West 1994; Wurst *et al.* 2004; Ueda *et al.* 2013; Simon *et al.* 2017). These effects likely occur due to alterations to plant defence and nutrition during AMF colonisation (Wurst *et al.* 2004; Meir & Hunter 2018b), but can depend on the level of AMF colonisation of the plant host (Tomczak & Muller 2017; Maurya *et al.* 2018; Meir & Hunter 2018a). As aphid herbivory may impose top down effects upon AMF colonisation via the shared host plant (Babikova *et al.* 2014a; Meir & Hunter 2018a), these top down and bottom up effects may modulate the outcome of the other, potentially resulting in above-below-ground feedback loops. Aside from impacting multitrophic interactions, if aphids influence AMF colonising the plant, this could alter how AMF effect plant nutrient uptake and abiotic tolerance. Thus, elucidating how AMF respond to top down effects is important in understanding AMF in multitrophic contexts such as agricultural systems. However, current knowledge on how AMF respond to aphids sharing the host plant is limited to the intraradical AMF colonisation of the plant root (Babikova *et al.* 2014a; Vannette & Hunter 2014; Maurya *et al.* 2018; Meir & Hunter 2018a). Extraradical structures are an important feature of AMF: AMF external hyphae can be directly related to AMF derived plant nutrient acquisition (Leigh, Hodge & Fitter

2009; Barrett *et al.* 2011), and AMF can carry signals of aphid attack to neighbouring plants via external hyphal networks (Babikova *et al.* 2013a).

As well as the physiological charactersitics of AMF, the structure of the AMF community colonising the plant host is important in determining AMF function: Small, artificially selected AMF communities reveal that the level of protection the AMF provides for the host plant against biotic stressors is related to AMF identity (Pozo *et al.* 2002; Sikes, Cottenie & Klironomos 2009; Malik, Dixon & Bever 2016), and certain AMF species may deliver more or less nutrients to their plant hosts (Jansa, Smith & Smith 2008; Leigh, Hodge & Fitter 2009). Moreover, soil community transfer experiments suggest AMF community determines nutrient acquisition and plant growth responses (Williams, Birkhofer & Hedlund 2014; Manoharan *et al.* 2017; Jiang *et al.* 2018). However, the function of soil transfer intolerant AMF taxa can be obscured in these studies (Sykorova *et al.* 2007) and it has been suggested that their function may be investigated by correlating the abundance of AMF taxa *in situ* with environmental factors in the field (Partida-Martinez & Heil 2011; Bainard *et al.* 2014; Jin *et al.* 2017). Moreover, whilst large vertebrate grazing may affect AMF communities (Ba *et al.* 2012; Guo *et al.* 2016), and insect herbivory may alter below ground ectomycorrhizal (Gehring & Bennett 2009), non-mycorrhizal fungi (Kostenko *et al.* 2012) and rhizosphere bacterial community characteristics (Kong *et al.* 2016), the impact of arthropod herbivory upon AMF communities is currently unclear.

The impact of herbivory on AMF can be contrasting (Barto & Rillig 2010), and phloem feeding aphids can increase or decrease the intraradical AMF colonisation of their host plant (Babikova *et al.* 2014a; Meir & Hunter 2018a). The C limitation hypothesis proposes that above ground removal of fixed C by herbivory will result in less C available belowground for the AMF (Wallace 1987), whilst low levels of herbivory can lead to more nutrients allocated below ground in an attempt for the plant to take up more nutrients for regrowth (Wamberg, Christensen & Jakobsen 2003). This is proposed to extend to the species richness and evenness of the AMF community, as higher levels of C could support more AMF species, and thus increased numbers and relative abundance of less competitive AMF species (Gange 2007). Indeed, AMF community diversity and evenness increased under low levels of above ground vertebrate grazing, and decreased under higher levels (Ba *et al.* 2012). However, herbivory of pinyon pine (*Pinus Cembroides* subspp.) by the needle feeding arthropod *Matsucoccus acalyptus* (Herbert) altered the identity of taxa in the ectomycorrhizal community (community composition), rather than species richness (Gehring & Bennett 2009).

As well as AMF communities being driven by C availability, plants could potentially select for AMF partners: As AMF communicate signals of above ground herbivory between plant hosts (Babikova *et al.* 2013a) signalling between the plant and AMF must occur under

herbivore pressure, and certain herbivore induced plant exudates may also be involved in the recruitment of protective beneficial microbes (Pineda, Kaplan & Bezemer 2017). Whitefly (*Bemisia tabaci* Genn.) feeding on pepper (*Capsicum annuum* L.) altered rhizosphere bacterial communities and using the model arthropod *Galleria mellonella* (L.), it was shown that these communities had increased abundance of arthropod killing microbes (Kong *et al.* 2016). Furthermore, vertebrate herbivore grazing of the perennial bunchgrass *Themeda triandra* Forssk. caused the plant to associate with an AMF community that provided defoliation tolerance (Gonzalez *et al.* 2018). Whilst it may be difficult to disentangle the above mechanisms of soil community selection, investigating the effects of aphids on the plant host and corresponding AMF community structure may provide a starting point to answer whether herbivory selects for specific AMF taxa or communities via changes to the host plant.

However, many AMF species are difficult to culture in pot systems (Ohsowski *et al.* 2014), and the majority of agricultural systems are 'conventionally' managed using tilling, chemical fertiliser, pesticide and fungicide regimes which often result in low diversity and distinct AMF communities (Jansa *et al.* 2002; Wetzel *et al.* 2014; Hartmann *et al.* 2015; Manoharan *et al.* 2017). Thus, a conventionally managed system was selected in the current study, allowing agriculturally relevant AMF communities that are tolerant to such practices to be investigated. Furthermore, Glomeromycotina comprise one of many fungal phyla that colonise or associate with plant roots, including other endophytic mutualists (Murphy, Doohan & Hodkinson 2015; Lugtenberg, Caradus & Johnson 2016). As some of these other fungi can influence aphid performance (Hartley & Gange 2009; Battaglia *et al.* 2013), the AMF community must be placed in the context of the wider fungal community, as non-AMF fungal community composition can also impact aphid performance (Kos *et al.* 2015).

This study aims to investigate the impact of aphid herbivory upon the intraradical, extraradical and community characteristics of AMF colonising the shared host plant, and also place community responses in the context of the wider fungal community. Furthermore, to attempt to elucidate the roles of agriculturally relevant AMF in the field, the relationship between the abundance of AMF taxa and above ground plant nutrition is also investigated. Specifically, the following hypotheses were tested: 1) Aphid herbivory of the host plant will depress plant growth and nutrient status. 2) Because of this impact on the plant, aphid herbivory will reduce intraradical AMF colonisation of the plant root, and the level of extraradical hyphae in the soil. 3) Aphids will also impact root fungal alpha diversity; a) reducing the number of AMF and total fungal species in the root, and b) decreasing the evenness of these communities, whilst c) impacting the relative abundance of AMF taxa. 4) Aphid feeding will also impact root fungal beta diversity,

leading to unique AMF and total fungal community compositions in plant roots. Finally, it is predicted that 5) AMF taxa abundance in an agricultural system will relate to above ground plant nutrition.

4.2 Methods

4.2.1 Site selection

Spring barley (*Hordeum vulgare* L., cultivar: Planet) was drilled into silty clay loam over chalk (Holly Hedge Field, Towthorpe, North Yorkshire, YO25 3HF, SE908625) on 15/03/17. Average soil characteristics of the whole field were sampled on 12/05/2017 and analysed by NRM Laboratories (Berkshire, UK), and are presented in the Appendix (Table 15). The field had been conventionally cropped with wheat and oats for the previous five years and treated with conventional agrochemical inputs throughout the duration of the study (Table 15; Appendix).

4.2.2 Aphid treatments

On the 21/04/17, lidless and bottomless PVC boxes (40 cm X 40 cm X 25 cm) were inserted 2-3 cm below the surface of the soil around sections of developing three leaf stage seedlings, averaging 26 ± 1.7 S.E. plants per box. This shallow insertion, so as not to disturb plant roots, allowed an aphid impermeable seal to form between the soil and a cage structure. The interior of each box thus formed an experimental ‘plot’. Experimental plots were assigned to ‘+Aphid’ or ‘-Aphid’ treatments and arranged in a randomised block design. As the site lay on a North Western slope, plots were set out in two rows perpendicular to the slope, in the North East direction. The location of the plots in the North East and North West direction were coded as the X and Y coordinate (respectively) of each plot within the field site. This attempted to account for any locational environmental gradients within the site, and are referred to as the X and Y axis plot location hereafter (Figure 9).

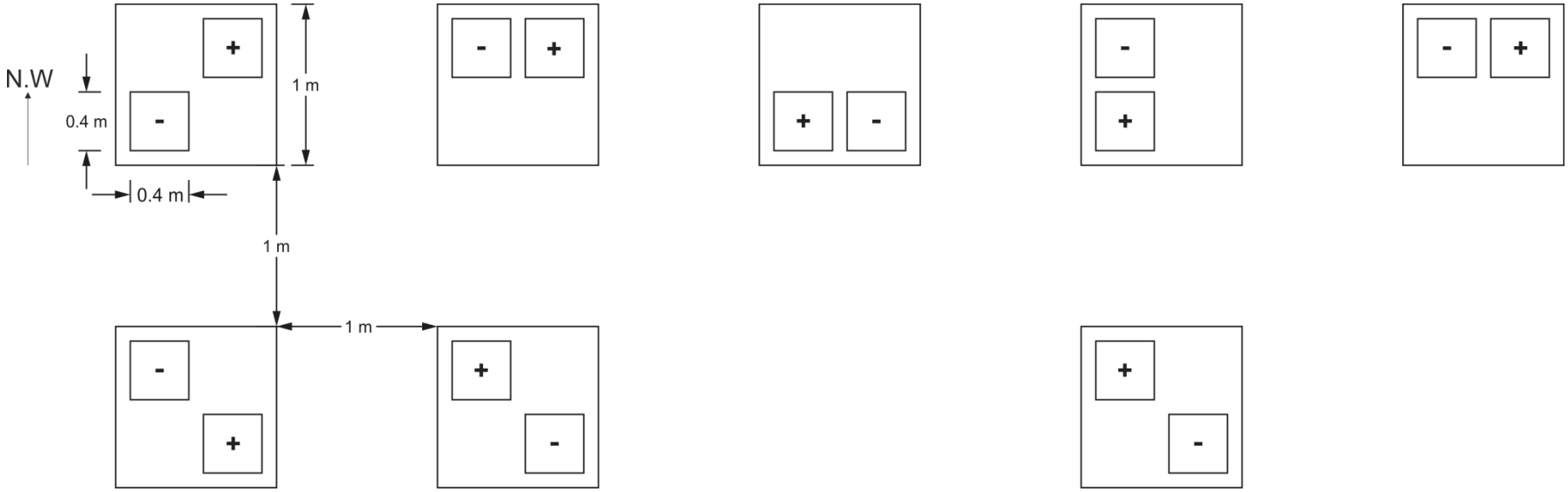

Figure 9. Field plot layout (3 m x 9 m). Plots of barley infested (+) or not infested (-) with aphids were arranged randomly in space within one of eight blocks (large squares) which were situated 1 m from each other. Plots were assigned a quarter of each block at random in order to reduce any positional bias within each block. The field site stood on a NW slope (Y axis of the site), and two rows were arranged perpendicular to this slope (in the NE direction; X axis of the site).

One week later (28/04/17) cages were constructed to cover each plot. The frames of the cage consisted of wooden posts inserted 20 cm into the soil and were attached to the interior of the PVC box. The cage extended 90 cm above the base of the soil and was covered with polypropylene horticultural fleece (Figure 10). English grain aphids (*Sitobion avenae* (F.)) (a single genotype, originally supplied by Koppert, Holland) were cultured on barley plants (cultivar Quench) at 20°C. From these cultures, ten 4th instar adults were taken at random and added to each +Aphid plot. All experimental plots (including -Aphid) were sealed with cages. *S. avenae* populations usually peak in the late summer months (Blackman & Eastop 2000), however, aphids and cages were added to the crop in the current study earlier than this to stop the natural ingress of aphids into the plots. Initially, eight replicates of each treatment were set up, although one +Aphid replicate was discarded during the study due to damage to the cage caused by high wind speeds.

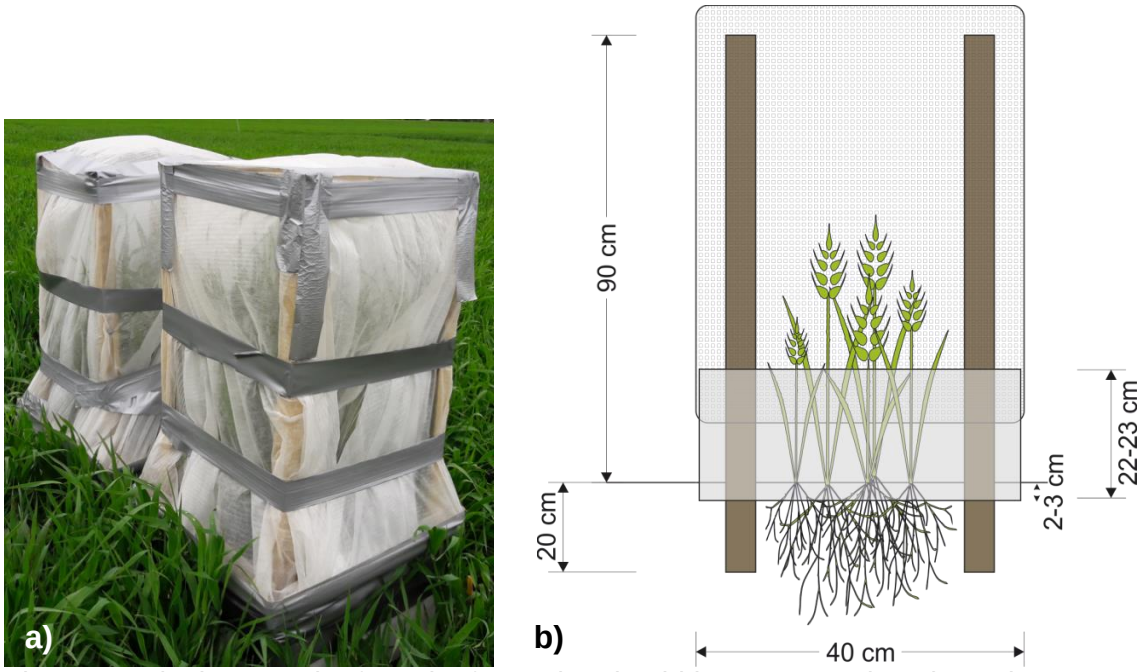

Figure 10. a) Photograph of a pair of completed aphid cages covering plots taken on 17/05/17. b) Schematic of cage design used to prevent aphid movement between the plots. Wooden posts reaching 90 cm high were inserted 20 cm into the ground to support a cage of polypropylene horticultural fleece sealed to a 40 X 40 cm PVC box reaching 22-23 cm above ground.

4.2.3 Harvest

Plots were harvested on the 13/06/17, 46 days post aphid addition. The number of aphids on five tillers chosen at random within each plot were recorded, and plots were dug out to 20 cm soil depth before storage at 4°C overnight. Three soil cores at each plot location were taken with a 2 cm diameter cheese corer between 20 and 30 cm soil depth. These

were processed to assess for the fungal hyphal length density (HLD) in the soil (Staddon, Fitter & Graves 1999). After storage, the aphids were discarded and plant roots were separated from the soil and washed whilst still attached to the above ground biomass. Only those roots visibly attached to a plant were stored at -20°C for DNA extraction or in 40 % ethanol for the staining of fungal structures. The above ground plant material was oven dried at 70°C for at least 96 hours and the total number of plants, tillers and fertile tillers were recorded. The above ground plant material for each plot was separated into a combined stem and leaf fraction, referred to as stem, as well as a separate grain fraction. It should be noted that due to the diffuse material of the stem fraction this was only able to be measured to the nearest gram. These fractions were homogenised in a kitchen blender (Igenix ig 8330, Ipswich, UK) before ball milling to a fine powder (Retsch MM400, Retsch GmbH, Haan, Germany). Resulting material was analysed for C and N ratios via a Elementar Vario El Cube (Elementar, UK, Ltd, Stockport, UK) and pelleted for X-ray fluorescence (XRF) analysis (Thermo Scientific™ portable X-ray fluorescence analyser) to determine P and Si concentrations as described by Reidinger, Ramsey and Hartley (2012).

The roots stored in 40 % ethanol were stained for fungal structures via the acetic acid–ink staining method (Vierheilig *et al.* 1998), modified to contain a 45 minute clearing step in 10 % KOH at 70°C and a 35 minute staining step using 5 % acetic acid, 5 % Pelikan® Brilliant Black ink and 90 % deionised water. Structures were assessed under a Nikon 50i eclipse microscope (Nikon UK Ltd, Surrey, UK) under 200 X magnification. As fungi other than AMF colonise plant roots in natural systems, a method that calculates both the most conservative (RLC min) and least conservative (RLC max) estimation of AMF RLC was used (Brundrett, Melville & Peterson 1994).

4.2.4 DNA extraction, PCRs and sequencing

Frozen root material was lyophilised for 36 hours and ball milled to a fine powder. The DNA was extracted from this material using a DNeasy PowerPlant Pro Kit (QIAGEN N.V, Venlo, Netherlands) according to the manufacturer's instructions except, that to increase the DNA yield, the DNA solution was eluted twice through the membrane in the final step. DNA concentrations were assessed (NanoDrop™ 8000 Spectrophotometer Thermo Fisher Scientific) and diluted to 20 ng μl^{-1} before use in the following PCR.

Two regions (amplicons) of fungal ribosomal DNA were amplified via PCR (Table 16; Appendix): An amplicon that captures the diversity of the entire fungal community (total fungi), and an amplicon that captures AMF specific diversity at a higher resolution and species coverage (AMF specific). After initial amplification via primary PCRs, secondary PCRs attached Illumina sequencing barcodes. For all PCRs the reaction consisted of 0.5

µl DNA, 0.1 µl of forward and reverse primers (20 mM) and 12.5 µl BioMix™ Red (Bioline, London, UK) made up to 25 µl reaction volume with molecular grade dH_2O. PCRs were carried out using a T100™ Thermal Cycler (BioRAD, Hercules, California, U.S.A.). The PCR products of the secondary PCRs were purified using a QIAquick PCR Purification Kit (Qiagen) and the purified concentrations were measured using a Qubit ® 3.0 fluorometer (Thermo Fisher Scientific™, Waltham, Massachusetts, U.S.A.). To mix the products of the two amplicons at equimolar concentrations to reduce sequencing depth bias during simultaneous DNA sequencing, these products were lyophilised overnight and re-suspended in molecular grade H_2O to achieve desired concentrations. Quality control and library preparation was carried out by the University of York Bioscience Technology Facility, and the resulting samples were sequenced using an illumina MiSeq system (illumina, San Diego, California, U.S.A.): Breifly, unique barcode sequences (Nextera XT index primers, illumina) were added onto amplicons tagged with illumina adapter sequences via PCR. Amplicons were then purified and pooled at equimolar ratios and then diluted and denatured. Samples were spiked with PhiX library spikes (illumina) for added sequence variety to enhance the distinguishing of floursecent signals of clusters during sequencing. Samples were run on a MiSeq 600 cycle kit (illumina).

4.2.5 Bioinformatic analysis

The raw forward and reverse reads were merged together resulting in a total of *c.* 1.5 million reads which were processed using QIIME2 (https://qiime2.org) (Caporaso *et al.* 2010). Reads were stripped of their primer and barcoding sequences and untrimmed reads were discarded (0.9 %). Reads from AMF specific amplicons were truncated to 270 bp due to estimated sequence quality drop off, whilst total fungal amplicons were not truncated. Reads were then dereplicated and clustered into operational taxonomic units (OTUS) using Usearch10 (Edgar 2010) with 97 % similarity. The resulting 516 Total Fungi amplicon OTUs were BLASTed against the UNITE ITS (Koljalg *et al.* 2013) database and eight non-fungal OTUs were removed, resulting in 509 total fungi OTUs, with the total read number per sample ranging from 53,333 to 129,338. Samples were normalised to 70,000 reads per sample using Usearch10's 'norm' function. Identical virtual taxon accessions according to the UNITE database were merged together yielding the 155 total fungi amplicon OTUs used in subsequent analysis.

A total of 27 OTUs were clustered together for the AMF specific amplicon. These were BLASTed against the maarjAM database (Opik *et al.* 2010) accessed on 13/06/18 and five OTUs with less than 96 % coverage and or similarity to taxa in the database were discarded. This resulted in a total read number from 12,035 to 21,706 per sample. Reads per sample were normalised to 16,500. AMF virtual taxonomic (VT) identities were assigned to the OTUs and a phylogenetic tree of the sequences was built to identify

identical VT accessions. Identical VTs were merged together resulting in 15 OTUs used in subsequent analysis. VTs were assigned according to the greatest BLAST coverage and similarity, however where OTUs could not be assigned to a single VT, VTs were labelled as unassigned.

4.2.6 Statistical analysis

Alpha diversity metrics (OTU/VT richness, Shannon's Index (e), Peilou's Evenness and Simpson's Diversity) were calculated using Usearch10 and all subsequent analyses were carried out in R version 3.3.2 (2016-10-31) (R Core Team 2016). To test the effect of aphid presence on AMF and total fungi alpha diversity metrics, AMF family relative abundance, and plant and AMF physiological characteristics, aphid presence was used as an explanatory variable in a linear model with plot location in the X and Y axes of the field site used as covariates, using the packages "lme4", "lmerTest", "lsmeans", and "car". As it was not feasible to test the effect of aphid presence on the relative abundance of the large number of families of the entire fungal community, the presence of fungal OTUs/VTs that predict either aphid treatment were identified via indicator species analysis using the "'indicspecies" package. Due to poorer identification and estimation of abundances (Berruti *et al.* 2017), AMF taxa identified in the analysis of all fungi were excluded from indicator species analysis. The effect of aphid presence and plot location on community composition between samples as measured via Bray-Curtis dissimilarity (Beta diversity) was analysed using a PERMANOVA via the 'Adonis' function in the "Vegan" R package. This was visualised via Non-metric multidimensional scaling (NMDS) and relationships between community composition and plant and AMF physiological characteristics were tested by applying the 'envfit' function to the NMDS. Possible relationships between AMF family relative abundance and plant and AMF physiological characteristics were tested via Pearson's correlation, the threshold for significance for correlations was set as $P = 0.05$ (Bainard *et al.* 2014; Guo *et al.* 2016; Xu *et al.* 2017; Yao *et al.* 2018). To reduce the effects of over dominance by one AMF taxon in AMF family relative abundance (RA) correlations, the RA of the most dominant taxa was calculated as normal, and for all other AMF families the RA was calculated using data excluding the most dominant taxa.

4.3 Results

4.3.1 Effect of aphid presence upon plant and AMF physiology

The mean number of aphids per tiller ± S.E in '+Aphid' plots was 24.9 ± 8.2, and ranged between 1.8 to 75, whilst no aphids were present in any '-Aphid' plots. Aphid presence significantly increased the stem N:P ratio of plants, and there was a trend of the grain N:P ratio also increasing. No other plant biomass or nutritional characteristics were affected by the aphid treatment, although grain P concentration and N:P ratio was associated with the

position of the plot in the X axis of the site (Table 9). Aphid treatment did not significantly impact any AMF physiological structures, although there was a trend of increased vesicle levels in the roots of plants hosting aphids, and vesicle levels were associated with the position of the plot in the Y axis (Table 9).

Table 9. Mean (± S.E) above ground plant biomass and nutrient concentrations, and AMF physiological characteristics of experimental barley plots infested with or without aphids, using the location of the plot in the X and Y axis of the site as a model covariate.

					Plot location covariate			
			Aphid presence		X axis		Y axis	
	-Aphid	+Aphid	$F_{1,11}$	*P*	$F_{1,11}$	*P*	$F_{1,11}$	*P*
Plot Plant No.	27.6 ± 3.2	25.1 ± 1.8	0.439	0.521	0.085	0.777	0.764	0.401
Plot Fertile Tiller	48.3 ± 4.7	51.1 ± 5.8	0.164	0.693	0.353	0.565	0.647	0.438
Plot Total Tiller	71.4 ± 4.5	67.3 ± 5.0	0.440	0.521	1.99	0.186	0.460	0.512
Plot Stem DW	57.13 ± 4.73	56.86 ± 3.50	0.002	0.963	0.097	0.761	1.731	0.215
Plot Grain DW	10.90 ± 1.38	10.87 ± 0.47	<0.001	0.982	0.244	0.631	0.024	0.880
Mean Tiller DW	0.95 ± 0.06	1.02 ± 0.05	0.985	0.342	1.706	0.218	0.767	0.399
Mean Grain DW	0.22 ± 0.01	0.225 ± 0.03	0.007	0.934	0.182	0.678	0.967	0.347
Grain [P]	3.63 ± 0.14	3.49 ± 0.10	0.899	0.363	5.122	**0.045**	0.059	0.813
Stem [P]	2.46 ± 0.10	2.46 ± 0.14	<0.001	0.996	0.030	0.865	0.040	0.845
Grain [N]	17.50 ± 0.52	18.38 ± 0.43	1.734	0.215	0.719	0.415	0.154	0.702
Stem [N]	18.16 ± 0.71	19.08 ± 0.89	0.664	0.432	0.139	0.716	0.035	0.855
Grain [Si]	10.30 ± 0.55	9.43 ± 0.58	1.239	0.289	0.443	0.519	0.031	0.864
Stem [Si]	9.69 ± 0.70	8.57 ± 0.48	1.599	0.232	0.005	0.943	0.077	0.786
Grain [C]	418.65 ± 1.89	421.68 ± 1.74	1.324	0.274	0.194	0.668	0.025	0.876
Stem [C]	420.84 ± 2.13	420.13 ± 2.16	0.055	0.819	0.370	0.556	<0.001	0.979
Grain C:N	24.05 ± 0.72	23.0 ± 0.62	1.194	0.298	0.442	0.520	0.102	0.756
Stem C:N	23.38 ± 1.06	22.28 ± 0.97	0.574	0.465	0.343	0.570	0.009	0.926
Grain N:P	4.85 ± 0.21	5.28 ± 0.16	4.736	0.052·	10.591	**0.008**	0.376	0.552
Stem N:P	7.40 ± 0.11	7.80 ± 0.18	5.143	**0.045**	2.806	0.122	1.200	0.297

RLC Min	32.67 ± 3.14	33.48 ± 3.06	0.034	0.857	0.021	0.888	0.409	0.536
RLC Max	47.45 ± 3.71	49.91 ± 3.91	0.284	0.605	0.149	0.707	0.715	0.416
HLD	0.16 ± 0.03	0.19 ± 0.04	0.511	0.490	0.442	0.520	1.052	0.327
Arbuscule	29.20 ± 2.57	29.77 ± 3.56	0.019	0.893	0.139	0.716	1.078	0.322
Vesicle	2.75 ± 1.03	5.29 ± 1.78	3.296	0.097·	0.021	0.888	11.606	**0.006**

[ ] = concentration (mg g^{-1}), No.= Number, DW = dry weight (g), RLC = AMF root length colonisation where Min = most conservative estimate and Max = least conservative estimate, RLC values, arbuscule and vesicle are % of root length, HLD = Hyphal length density (m hyphae g^{-1} soil).

4.3.2 Effect of aphid presence on total fungal and AMF communities in the root

Across both aphid treatments, within the entire fungal community 155 ‘total fungi’ OTUs were identified from nine fungal phyla as well as OTUs which could not be assigned to phylum level. The highest abundance of sequences were assigned to Ascomycota (90.2 %), followed by Basidiomycota (5.3 %), unclassified fungi (3.5 %), Glomeromycota (0.83 %), and Chytridiomycota (0.08 %), with sequences from Rozellomycota, Mortierellomycota, Entomophthoromycota, Mucoromycota and Zoopagomycota contributing to less than a combined 0.1 % of sequence abundance. Within the AMF specific amplicon, 12 OTUs were assigned to VTs whilst three OTUs could not be assigned to a singular VT (Table 17; Appendix). These VTs belonged to 7 AMF families: Glomeraceae (6), Paraglomeraceae (1), Diversisporaceae (2), Ambisporaceae (1), Gigasporaceae (1), Archaeosporaceae (3) and Acaulosporaceae (1).

Aphid presence did not affect the number of species within the entire fungal community, but did increase its evenness (Table 10). Whilst aphid presence did not affect any AMF specific alpha diversity metrics, including species richness, the Simpson’s diversity of the AMF specific community was linked to plot location on the X axis of the field site (Table 10).

Aphid presence had no effect on the relative abundance (RA) of Glomeromycotina (AMF) reads within the entire fungal community, although within the AMF specific amplicon the RA of the Gigasporaceae family tended to increase when aphids were present. The RA of AMF reads within the entire fungal community, and the RA of the Gigasporaceae and Ambisporaceae families present in the AMF specific community were associated with the location of the plot in the X axis of the site (Table 11).

Table 10. Mean (± S.E) Alpha diversity metrics for 'Total Fungi' amplicon OTUs, and 'AMF specific' amplicon VTs from barley plots without or without the presence of aphids, using the location of the plot in the X and Y axis of the site as a model covariate.

					Plot location covariate			
			Aphid presence		X axis		Y axis	
	-Aphid	+Aphid	$F_{1,11}$	*P*	$F_{1,11}$	*P*	$F_{1,11}$	*P*
Total fungi								
OTU richness	92.63 ± 2.49	89.43 ± 2.26	0.889	0.366	0.281	0.607	0.087	0.774
Shannon's Index	2.54 ± 0.09	2.75 ± 0.06	3.725	0.080·	0.008	0.932	0.170	0.688
Peilou's Evenness	0.56 ± 0.02	0.61 ± 0.01	5.09	**0.045**	<0.001	0.977	0.260	0.620
Simpson's Diversity	0.15 ± 0.02	0.11 ± 0.01	3.06	0.108	0.527	0.483	0.377	0.551
AMF								
VT richness	11.25 ± 0.34	11.14 ± 0.50	0.033	0.859	0.004	0.953	0.105	0.752
Shannon's Index	1.17 ± 0.11	1.20 ± 0.09	0.047	0.833	2.646	0.132	0.264	0.618
Peilou's Evenness	0.49 ± 0.04	0.51 ± 0.04	0.075	0.790	3.018	0.110	0.429	0.526
Simpson's Diversity	0.47 ± 0.05	0.44 ± 0.05	0.226	0.644	6.207	**0.030**	0.176	0.683

Table 11. Mean (± S.E) relative abundances (RA) of the AMF sequences within the 'Total Fungi' amplicon, and of AMF family sequences from the 'AMF specific amplicon', from experimental barley plots infested with or without aphids, using the location of the plot in the X and Y axis of the site as a model covariate.

					Plot location covariate			
			Aphid presence		X axis		Y axis	
	-Aphid	+Aphid	$F_{1,11}$	*P*	$F_{1,11}$	*P*	$F_{1,11}$	*P*
AMF RA	0.53 ± 0.29	1.18 ± 0.63	1.450	0.254	5.447	**0.040**	0.145	0.710
Acaulosporaceae RA	0.02 ± 0.02	0	-	-	-	-	-	-
Ambisporaceae RA	3.21 ± 0.79	2.92 ± 0.71	0.123	0.732	8.111	**0.016**	0.0357	0.854
Archaeosporaceae RA	1.23 ± 0.41	0.63 ± 0.19	1.828	0.204	0.056	0.817	1.848	0.201
Diversisporaceae RA	3.72 ± 0.68	5.68 ± 2.52	0.767	0.400	2.241	0.163	0.198	0.665
Gigasporaceae RA	0.77 ± 0.20	1.48 ± 0.52	4.106	0.067·	13.560	**0.004**	0.553	0.473
Glomeraceae RA	85.44 ± 2.18	83.43 ± 2.30	0.420	0.530	0.778	0.397	<0.001	0.990
Paraglomeraceae RA	5.62 ± 1.32	5.86 ± 1.54	0.016	0.900	0.658	0.434	1.119	0.313

All but two AMF specific amplicon VTs were found in both aphid and no aphid treatments, however the exceptions were not strong indicators of aphid presence or absence (Table 12). Several Ascomycota taxa were strong predictors of the absence of aphids for the Total Fungal amplicon, whilst a Basidiomycota genus indicated aphid presence.

Table 12. Indicator species analysis of AMF specific amplicon VTs and Total Fungi amplicon OTUs as indicators of uninfested or aphid infested barley plots. Only Total Fungi amplicon OTUs which significantly indicate the aphid treatments are shown.

OTU/VT	-Aphid	+Aphid	IndVal	*P*
AMF specific				
Acaulospora VTX00030	1	0	0.35	1.00
Glomus VTX00199	0	1	0.49	0.35
Total Fungi				
Ascomycota; Pseudeurotiaceae	1	0	0.92	**0.006**
Ascomycota; Helotiales	1	0	0.88	**0.046**
Ascomycota; Halosphaeriaceae	1	0	0.87	**0.032**
Basidiomycota; Cystobasidiaceae	0	1	0.81	**0.029**

The difference in Total Fungi amplicon community composition between sites measured as Bray-Curtis dissimilarity (beta diversity) was not significantly affected by aphid presence or plot location in the X or Y axis of the field site (PERMANOVA: $F_{1,11} = 1.78$, $P = 0.129$; $F_{1,11} = 1.74$, $P = 0.136$; $F_{1,11} = 0.03$, $P = 0.808$ respectively). However, community composition correlated with plot location on the X axis ($R^2 = 0.46$, $P = 0.028$) and grain P concentration ($R^2 = 0.46$, $P = 0.034$; Figure 11).

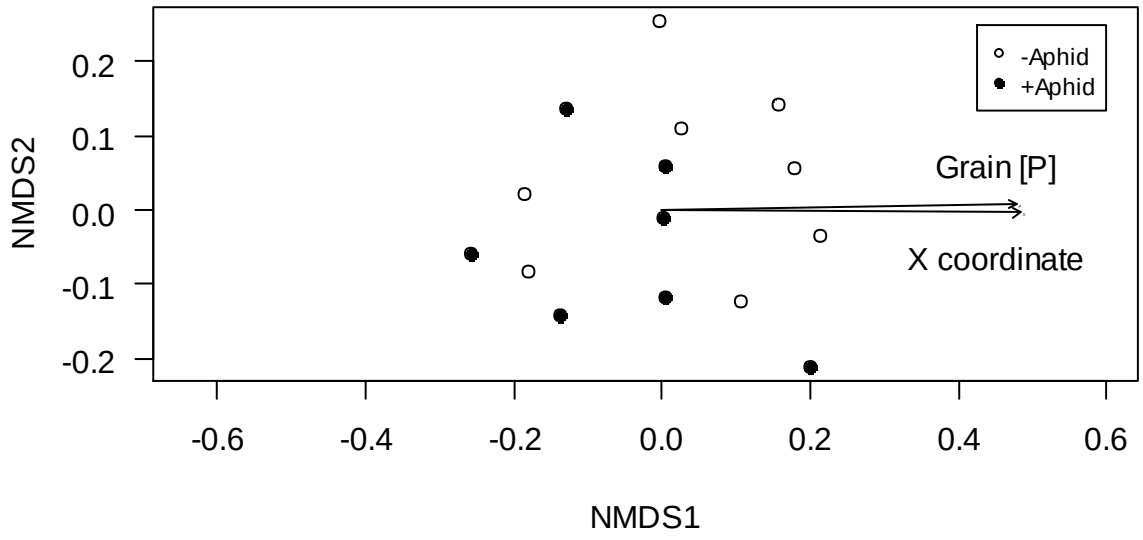

Figure 11. Total Fungi community composition: Non Metric Multidimensional Scaling (NMDS) of Total fungi community distribution based on Bray-Curtis dissimilarity (stress = 0.158) obtained from the abundance of 'Total fungi amplicon' OTUs under '+Aphid', or '-Aphid' treatments. Total fungi community composition was significantly associated with the barley P concentration (mg g^{-1}) in the plots (Grain [P]), and the location of the plot in the X axis (X coordinate).

The community composition of AMF specific VTs in the roots of barley plants between sites was not affected by the presence of aphids above ground or the location of the plot in the Y axis ($F_{1,11}$ = 0.71, P = 0.453; $F_{1,11}$ = 0.473, P =0.578 respectively), but was affected by the location of the plot in the X axis of the field site ($F_{1,11}$ = 8.83, P = 0.010). Moreover, the environmental factors of stem Si and location in the field were significantly correlated with the community composition (R^2 = 0.39, P = 0.042 and R^2 =0.40, P = 0.047, respectively; Figure 12).

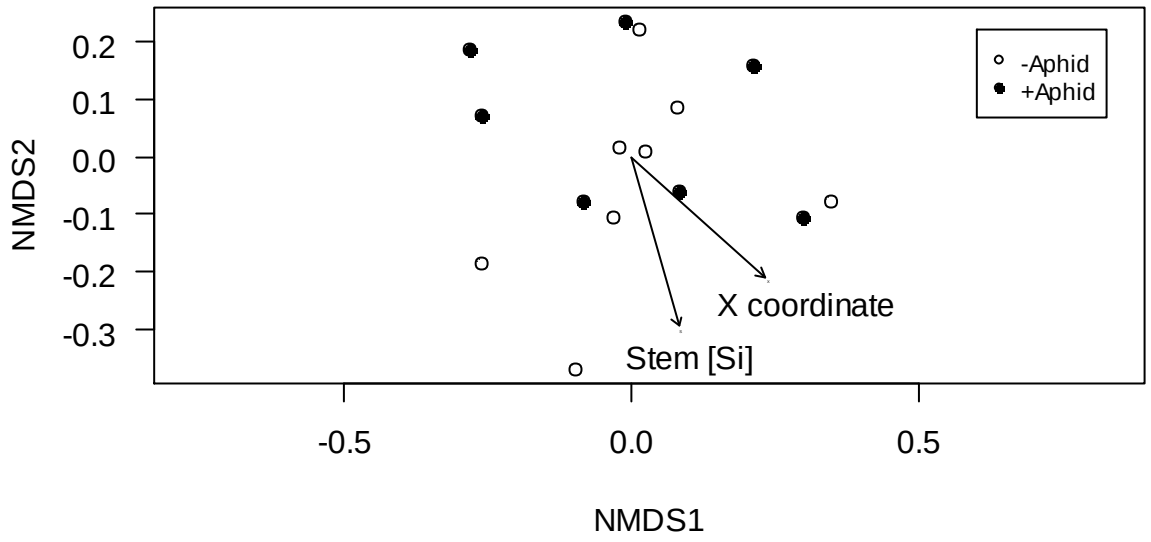

Figure 12. AMF specific community composition: Non Metric Multidimensional Scaling (NMDS) of the AMF community distribution based on Bray-Curtis dissimilarity (stress = 0.154) obtained from the abundance of 'AMF specific amplicon' VTs under '+Aphid', or '-Aphid' treatments. AMF specific community composition was significantly associated with the barley stem silicon Si (mg g^{-1}) concentration in the plots (Stem [Si]), and the location of the plot in the X axis (X coordinate).

4.3.3 Relationships between AMF family VT abundance and plant nutrition

To investigate associations between above ground crop nutrition and the identity of agriculturally relevant AMF taxa *in situ*, plant and AMF physiological traits were correlated with the abundance of AMF families in the field. The RA of the most abundant family of AMF found in the barley roots, Glomeraceae, was positively correlated with the most conservative estimate of RLC and arbuscule levels, but was negatively correlated with stem biomass and the concentration of C in the grain of host plants (Figure 13). The abundance of Gigasporaceae was also positively correlated with the most conservative estimate of RLC as well as arbuscule number. Archaeosporaceae abundance positively correlated with stem P and Si concentrations and negatively with stem C (Figure 13). All AMF physiological characteristics measured were negatively associated with the total biomass of the stems (Figure 13).

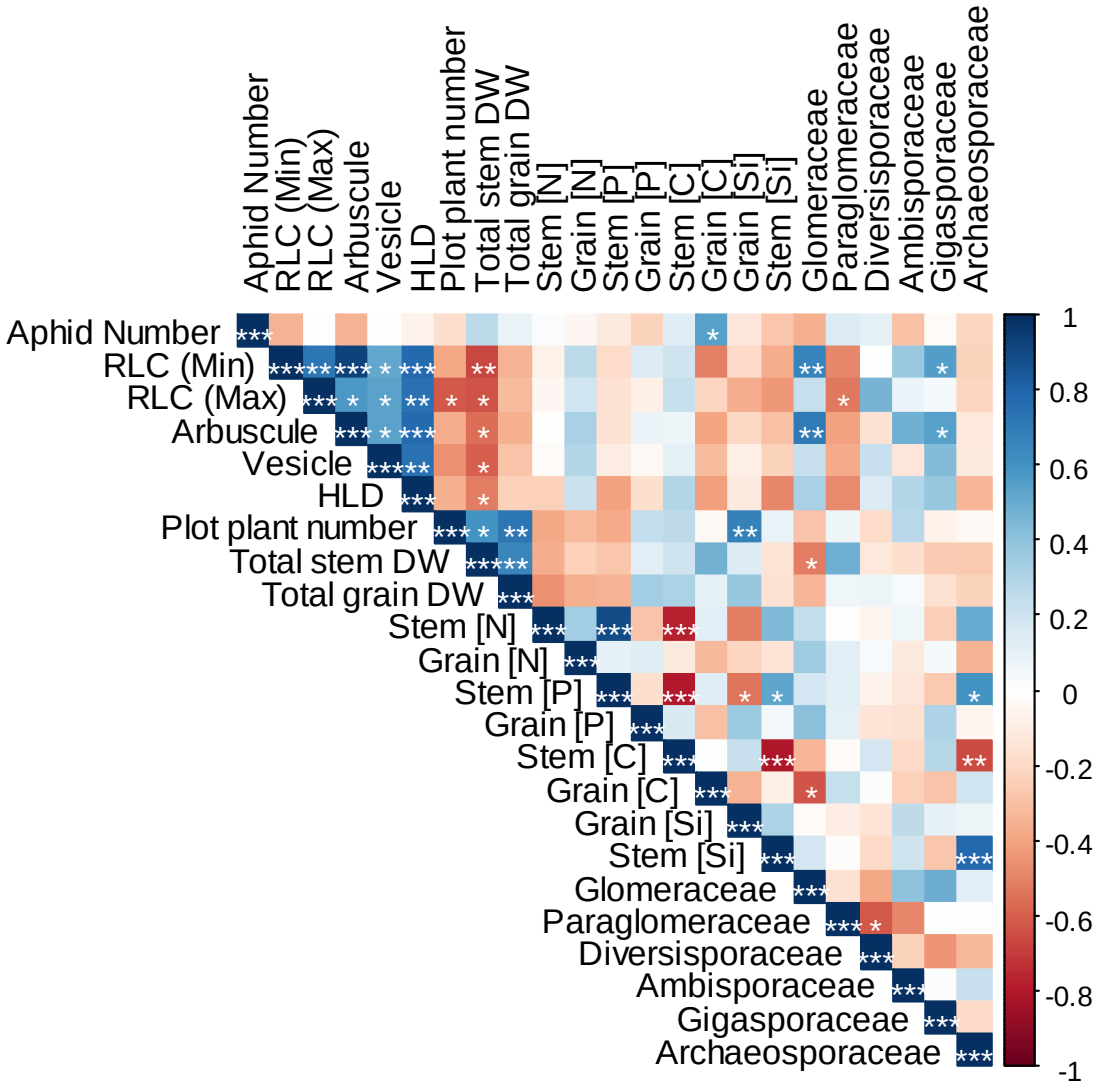

Figure 13. Matrix of Pearson's correlations of AMF VT family relative abundances and AMF and plant physiological traits. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Scale value = correlation coefficient. [ ] = concentration (mg g^{-1}), DW = dry weight (g), RLC = AMF root length colonisation where Min = most conservative estimate and Max = least conservative estimate, RLC values, arbuscule and vesicle are % of root length, HLD = Hyphal length density (m hyphae g^{-1} soil).

4.4 Discussion

This study aimed to investigate the impact of aphid herbivory upon AMF in a conventionally managed agricultural system. To capture the impacts of a disturbance on AMF, it is important to measure both fungal physiological characteristics, as well as AMF diversity and community composition (van der Heyde *et al.* 2017a) measured here through analysis of an AMF specific amplicon. As AMF share the host plant with other fungi, the context of the entire fungal community was also measured using a less precise, but wider encompassing amplicon. It was hypothesised that via supressing plant nutrition

and growth, aphid feeding would lead to negative impacts upon AMF physiology, as well as fungal species richness and evenness. It was also proposed that the effects of aphids would also impact the community composition of the fungal communities, and the abundance of AMF taxa. Aphid presence had less of an impact on plant physiology and AMF community structure than the location of the shared host plant in the field, although there was a trend of the abundance of AMF vesicles, and the abundance of the Gigasporaceae family to increase when aphids fed upon the shared host plant. Within the context of the entire fungal community, AMF were also affected by location rather than aphid presence, although aphid feeding on host plants did increase the evenness of the total fungal community.

4.4.1 Effects of aphid herbivory on the plant and AMF physiology

Contrary to the first hypothesis, *S. avenae* had little effect on the above ground nutrition of barley in the field, although aphid presence tended to increase the above ground plant N:P ratio, possibly due to nutrient re-allocation caused by aphid feeding (Sandstrom, Telang & Moran 2000; Thompson & Goggin 2006; Nowak & Komor 2010). Moreover, aphid presence did not reduce above ground biomass in the current study. It is possible that as the aphids in the current study were cultured in controlled conditions, the vectoring of plant viruses by *S. avenae* that contributes to yield loss in cereals (McKirdy, Jones & Nutter 2002) did not occur. It was hypothesised that any negative impact of aphids upon above ground plant physiology would be passed on to the intraradical colonisation of the shared host plant by AMF, as well as the production of external hyphae in the soil. As no negative impact occurred, this may explain why AMF RLC and hyphal length density were not affected by aphid presence on the host plant. However, there was a trend for aphid herbivory increasing the proportion of vesicles in plant roots. Defoliation can influence the proportion of vesicles in plant roots (Garcia & Mendoza 2012). As vesicles are lipid storage organs in AMF, and AMF derive lipids from the host plant (Keymer & Gutjahr 2018), this suggests that *more* fixed C is available to the AMF via the plant under aphid herbivory. Aphids can increase and decrease below ground respiration depending on plant growth stage, potentially due to alterations in C availability to soil microbes (Vestergard, Bjornlund & Christensen 2004). In the current study, more C may be made available to the AMF in order to increase nutrient uptake for above ground regrowth (Wamberg, Christensen & Jakobsen 2003).

4.4.2 Aphid herbivory on AMF and total fungal communities

The third hypothesis proposed that the effects of aphid feeding upon the shared host plant would reduce AMF and total fungal species richness and evenness. The low richness of AMF species identified in the current study is similar to that documented in other conventionally managed barley monoculture systems (Manoharan *et al.* 2017), and as the

read depth achieved for the AMF specific amplicon is sufficient to capture AMF diversity (Vasar *et al.* 2017), it can be assumed that this is an accurate representation. Neither the number of species of AMF, nor the entire fungal community were impacted by aphid presence, reflecting the results of arthropod feeding upon pinyon pine associated ectomycorrhizal communities (Gehring & Bennett 2009). Moreover, aphid presence increased the evenness of the entire fungal community in the current study, contrary to expected. As well as the potential for intermediate levels of aphid herbivory leading to increased C allocation to the roots, aphids excrete honeydew as a result of their C rich diet of phloem sap which can be utilised as a C source by soil microbiota, thus shaping community structure and biomass (Katayama *et al.* 2014; Milcu *et al.* 2015). As more C sources become available in the root, it is possible that niches may enlarge, reducing the dominance of abundant taxa. However, it should be noted that aphid induced alterations to soil macroorgansisms can occur independently of honeydew C inputs (Sinka, Jones & Hartley 2009), and that soil microbes can be influenced by plant exudates in systems where honeydew does not reach the microbe (Kim, Song & Ryu 2016).

It was predicted that the abundance of AMF taxa within the AMF community would be impacted by aphid presence, and there was a marginal increase in the abundance of Gigasporaceae under aphid infestation of the host plant. A recent meta-analysis reveals that members of this family are tolerant to fertiliser input disturbances, suggesting a role aside from nutrient acquisition, perhaps in plant defence (van der Heyde *et al.* 2017b). Species indicator analysis may also identify taxa affected by treatments, however, as low abundance taxa score as poor indicators, this method may provide contrasting results to investigating relative abundances (Longa *et al.* 2017). No AMF VTs identified using the AMF specific amplicon were indicators of either treatment, but several total fungi amplicon OTUs were indicators of aphid presence or absence. Currently, it is unclear whether these organisms are responding to changes in nutrient availability, or whether the plant recruits these soil microbes in response to aphid feeding. It has been proposed that below ground microbial communities could be steered to better supress above ground pests, which could reduce the adverse environmental impacts associated with pesticide inputs (Pineda, Kaplan & Bezemer 2017), leading to more sustainable and resilient food production (Thirkell *et al.* 2017). Further study is required, however, to elucidate whether increases in the abundance of soil fungi in the current study results in reduced aphid performance, as aphid feeding can lead to the recruitment of soil bacteria that ultimately benefit the aphid (Kim, Song & Ryu 2016).

Changes to fungal identity within the communities of each treatment were not clear at the community composition level, perhaps as a longer period of top down pressure is required to impact community composition: the effects of grazing by large vertebrates upon AMF

community structure are strongly linked to the length of the pressure (van der Heyde *et al.* 2017a). However, as plant communities are removed annually in cereal systems, and aphid feeding is seasonal (Blackman & Eastop 2000), a relatively short window is available for these interactions to occur. Strong impacts of plot location upon fungal community composition and abundance could have contributed to masking top down effects on community composition. AMF communities may be associated with environmental gradients even over short distances (Horn *et al.* 2014), and as grain P concentration and plot location in the X axis are tightly linked to AMF abundance and community composition in the current study, this suggests that AMF communities followed nutritional gradients in the field site, as described in other systems (Bainard *et al.* 2014).

4.4.3 Associations between AMF and above ground plant nutrition in a field system

Although it is difficult to disentangle the response of AMF taxa to soil nutrient availability from that of the effects of AMF taxa upon plant nutrition, the final hypothesis predicted an association between *in situ* AMF family abundance and above ground plant nutrition. Interestingly, the most conservative estimate of RLC correlated to the abundance of Glomeraceae. This AMF family preferentially allocates biomass inside the root, perhaps avoiding mechanical disturbance commonly found in high till systems (Chagnon *et al.* 2013). Negative correlations between the abundance of Glomeraceae and stem biomass and also grain C concentration suggests that this preferentially intraradical growth may be associated with a reduced benefit for the plant. Although it is possible that plant density, which could confound associations with total plot plant biomass, may shape AMF communities (Sasvari & Posta 2010), the abundance of any family in the current study did not correlate with the number of plants within each plot. Moreover, the physiological characteristics of AMF were negatively associated with stem biomass, suggesting that the total amount of, and identity of AMF may be important in plant growth responses in the field. The abundance of a rarer family of AMF, Archaeosporaceae, was associated with above ground P and Si concentrations. Whilst the abundance and diversity of AMF have previously been associated with soil environmental characteristics (Bainard *et al.* 2014; Guo *et al.* 2016; Yao *et al.* 2018), here, the abundance of AMF taxa has been associated with plant nutrition. This highlights the importance of AMF identity in plant growth even in conventional systems. It is likely Archaeosporaceae drives the association of AMF community composition and stem Si, although whether this is due to increased uptake of Si due to AMF (Garg & Bhandari 2016; Frew *et al.* 2017a), or an artefact of AMF responses to soil pH (Bainard *et al.* 2014) requires further study.

4.4.4 Conclusions

Aphid herbivory increased the evenness of the entire fungal community within plant roots, and also tended to increase the level of vesicles and abundance of the AMF family Gigasporaceae. Whether these increases are due to increased C allocation below ground by plants attempting to increase nutrient uptake, or the active selection of fungal taxa in response to herbivory requires elucidation. Whether these changes in the below ground soil community feeds back into altered aphid performance is currently unclear, but the response of agriculturally relevant fungal communities to 'top down' effects of herbivory suggests that above-below ground community feedback could occur in agricultural systems. It is possible that the likely hood of a plant hosting AMF that respond to above ground herbivory or plant nutrition would increase in a more diverse AMF community. Even within the low AMF diversity of the current study however, the abundance of AMF families and rare taxa may be associated with above ground plant physiology and nutrition. This highlights the importance of linking mechanistic function to phylogeny in understanding how we can best utilise AMF in increasing the sustainability of modern agriculture, and that the importance of rare AMF taxa should not be overlooked. Further study should investigate whether above-below ground soil feedback or fungal taxa associations with plant nutrition can be used to increase the efficacy of soil fungi as alternatives to agro-chemical inputs. Whether the enrichment of soil fungal communities with taxa associated with host plant nutrition or pest presence impact these characteristics in agriculturally relevant systems should be tested.

5 Mycorrhizal induced resistance against an aphid is not mitigated by its facultative symbiont

5.1 Introduction

Interactions with microbes can aid multicellular organisms in the defence against natural enemies (Oliver *et al.* 2003; Hartley & Gange 2009; Mattoso, Moreira & Samuels 2012; Lauer & Hernandez 2015; Lagier 2016), and over time these interactions may evolve into intimate symbiotic associations that can become integral to how the host partners defend themselves (Ford & King 2016). However, natural enemies may also associate with microbial symbionts, allowing them to overcome the defence responses of their host or prey (Barr *et al.* 2010; Frago *et al.* 2017). In certain cases, these interactions can reduce the effectiveness of biological controls targeted at crop pests (Vorburger 2018). As microbes associated with the natural enemy (Barr *et al.* 2010; Kaiser *et al.* 2010; Chung *et al.* 2013) and microbes associated with the defending plant (Hartley & Gange 2009; Pineda *et al.* 2012) can both affect plant chemistry and defence signalling, it is likely that these herbivore and plant associated symbionts interact. However, little is known about how they act in concert, important in predicting the outcome of multitrophic interactions in natural systems and their use in the biological control.

Such symbiotic interactions are prevalent in many crop species including legumes (Babikova *et al.* 2014a), as well as phloem feeders such as aphids (Zytynska & Weisser 2016; Guo *et al.* 2017), which are detrimental to crop yeilds (Yencho, Getzin & Long 1986; Soroka & Mackay 1990; Zytynska & Weisser 2016). For example, herbivore association with the facultative secondary symbiont *Candidatus* Hamiltonella defensa Moran et al. (henceforth referred to as *H. defensa*) may attenuate plant defence signalling and volatile compounds that attract natural enemies of the herbivore (Su *et al.* 2015; Frago *et al.* 2017), and the colonisation of plant roots with arbuscular mycorrhizal fungi (AMF), which are obligate symbionts, may affect the performance of aphids sharing the same host plant (Gange & West 1994; Wurst *et al.* 2004; Babikova *et al.* 2014a; Simon *et al.* 2017).

It is proposed that AMF could be used as a biological control to supress pests in agricultural systems, reducing pesticide input requirements (Pineda, Kaplan & Bezemer 2017; Thirkell *et al.* 2017). AMF induced effects on aphid performance have previously been attributed to changes in the host plant quality as a food source (Wurst *et al.* 2004; Ueda *et al.* 2013; Tomczak & Muller 2017) as AMF may increase plant nitrogen (N) acquisition (Hodge & Storer 2015), a limiting nutrient in the diet of aphids (Butler, Garratt & Leather 2012). Moreover, AMF may improve plant phosphorus (P) uptake via AMF external hyphae extending P depletion zones that accumulate around the plant root (Smith *et al.* 2011).

Recently, AMF induced effects on aphid performance have also been associated with changes to plant defence (Babikova *et al.* 2014a; Meir & Hunter 2018a). However, the underlying plant defence signalling interactions induced by both AMF and aphids in the shared host plant remain unclear. During AMF colonisation, AMF and associated soil bacterial compounds are proposed to elicit plant defence signalling, including but not limited to the jasmonic and salicylic acid (JA/SA) pathways (Cameron *et al.* 2013; Perez-de-Luque *et al.* 2017). This can lead to the 'priming' of plant defences. During (plant defence) priming, a priming stimulus such as colonisation with a beneficial microbe causes the plant to enter a primed state. Entering this state can be associated with the slight induction of defence metabolites, but otherwise a defence response is only induced in response to pest attack. This defence response is stronger and/ or quicker than in an unprimed plant (Balmer *et al.* 2015; Martinez-Medina *et al.* 2016), and priming can play a key role in Mycorrhizal Induced Resistance (MIR) - the systemic induction of plant resistance by AMF colonisation (Mauch-Mani *et al.* 2017).

Whether priming occurs in AMF-aphid interactions was investigated in interactions between the pea aphid (*Acyrthosiphon pisum* (Harris)) and *Medicago truncatula* Gaertner (Maurya *et al.* 2018). Although AMF and aphids affected gene expression, a change in aphid performance could not be linked to an interaction between the two. As gene expression was captured 7 days post aphid addition, it was proposed that this time point could have missed key interactions between AMF and aphids earlier in the study. Indeed, interactions between beneficial bacterial soil microbes and aphids on plant defence have previously been captured within the first 48 hours of aphid infestation (Pineda *et al.* 2012).

The expression of genes regulated by SA pathways, such as pathogenesis related (PR) proteins, as well as those involved in JA pathways, including lipoxygenase (LOX) genes, are often used as a measure of primed plant defence responses (Song *et al.* 2015). AMF may also alter reactive oxygen species (ROS) metabolism in response to attackers (Vos *et al.* 2013), and can modulate genes involved in scavenging ROS, such as super oxide dismutase (SOD) (Mo *et al.* 2016) which can act as an early signal in plant defence pathways and directly damage attackers (reviewed in Nath *et al.* (2016)). However, it can be difficult to disentangle this priming phenomenon from changes to plant nutritional quality and constitutive defence induction during MIR (Mauch-Mani *et al.* 2017). Moreover, evidence suggests that AMF colonised plants may acquire increased amounts of silicon (Si) from the soil (Garg & Bhandari 2016), which can itself prime plants against phloem feeders (Yang *et al.* 2017a).

Aphids may also modulate the SA and JA signalling pathways of the shared host plant (Kusnierczyk *et al.* 2008; Schwartzberg & Tumlinson 2014). For example, plants induce defence responses upon the perception of the aphid as well as its associated obligate

primary symbiont *Buchnera aphidicola* (Chaudhary *et al.* 2014; Kaloshian & Walling 2016). Aphids combat this by secreting salivary proteins, or 'effectors', to interfere with the plant defence signalling process (Kettles & Kaloshian 2016; Mugford *et al.* 2016). Due to these effectors and the low levels of mechanical damage associated with phloem piercing and feeding, aphids commonly induce SA pathway plant defences, that are otherwise associated with biotrophic pathogens (Jaouannet *et al.* 2014). Aphid SA induction can supress JA related defence pathways (Schwartzberg & Tumlinson 2014), potentially via SA/JA antagonsim (Thaler, Humphrey & Whiteman 2012). JA based defences are usually associated with necrotrophic pathogens and leaf chewing herbivores (Berens *et al.* 2017), but are also effective against aphids (Cooper & Goggin 2005; Gao *et al.* 2007; Haas *et al.* 2018).

Thus, AMF and aphids could simultaneously interact with plant host defence signalling. There is scope for AMF to induce a heightened defence response against aphid attack, but it is also possible that AMF could influence aphid manipulation of the plant SA/JA antagonism, leading to increased susceptibility to aphids. Moreover, phloem feeding insects may harbour their own symbiotic associations that enhance plant defence manipulation. For example, in whitefly, the facultative symbiont *H. defensa* may suppress plant host JA signalling, increasing whitefly performance (Su *et al.* 2015), and this same species of symbiont in aphids supresses volatile production that would attract aphid natural enemies (Frago *et al.* 2017). However, harbouring *H. defensa* did not modify the response of the potato aphid (*Macrosiphum euphorbiae* (Thomas)) to AMF colonisation of *Solanum* spp. host plants (Bennett *et al.* 2016). The protection of an aphid against natural enemies can be dependent on *H. defensa* strain (Oliver, Moran & Hunter 2005) and thus may have been missed in this study. Nevertheless, aphids carrying *H. defensa* reduced the root biomass of *Solanum* spp. host plants (Hackett, Karley & Bennett 2013; Bennett *et al.* 2016), and thus aphid facultative symbionts may modulate plant resource allocation and nutrient acquisition, which could feed back into the performance of the aphid. The impacts of aphids on the multitrophic system may also extend to the intraradical colonisation of the host plant (Babikova *et al.* 2014a; Meir & Hunter 2018a). This multitrophic interaction is again proposed to occur via changes to the plants nutritional status (Wallace 1987) or plant defence signalling pathways (Babikova *et al.* 2014a). However, whether aphid facultative symbionts alter intraradical AMF colonisation is unclear. Furthermore, whether aphid feeding affects AMF external structures such external hyphae, which are important in AMF nutrient acquisition (Smith *et al.* 2011), is currently unknown.

Here we used two experiments to investigate the underlying mechanisms involved in AMF induced changes to *A. pisum* performance, and whether the presence and strain of *H.*

defensa within aphids alters this outcome. We hypothesised that 1) AMF may affect aphid performance by one of two mechanisms i.e. by a) altering the above ground nutritional status of the shared host plant or b) inducing changes in SA and JA dependent gene expression in the host plant. 2) We hypothesised that these AMF induced changes to aphid performance will be dependent upon the presence and strain of *H. defensa* carried by the aphid, and that 3) Aphids would alter the amount of both intraradical and extraradical AMF structures.

5.2 Materials and Methods

5.2.1 AMF-aphid-*H. defensa* interactions

5.2.1.1 Experimental system

The effects of AMF colonisation upon pea aphids (*Acyrthosiphon pisum* (Harris)) feeding upon broad beans (*Vicia faba* L.) are well characterised; AMF colonisation can increase or decrease aphid performance and carry signals of aphid attack between plant hosts in this system (Babikova *et al.* 2013a; Babikova *et al.* 2013b; Babikova *et al.* 2014a; Babikova *et al.* 2014b). Thus, these species form a suitable model system to investigate plant defence signalling and how *H. defensa* may modify AMF-aphid interactions. The pea aphid lines used in this experiment were originally generated by Melanie Smee at The University of York for a previous experiment: The aphid genotype was originally collected from *Medicago sativa* L. in Berkshire in 2010 and was originally infected with two species of facultative symbionts, *Candidatus* Hamiltonella defensa and *Candidatus* Fukatsuia symbiotica (also known as X-type or PAXS). The symbionts were removed using antibiotics following the protocol in McLean *et al.* (2011). To generate aphid lines with different strains of *H. defensa*, aphids were injected with haemolymph of aphids carrying different isolates of *H. defensa*, approximately four years before the experiments reported here. These donor aphids had all been collected from *M. sativa*: H_{236} in Buckinghamshire in 2010, and H_{207} and H_{216} in Lincolnshire in 2012. The infection status of the aphids was confirmed at the beginning of the current study using the protocol of Ferrari *et al.* (2012). A single species of AMF (*Funneliformis mosseae* (Nicolson & Gerd.) Walker & Schüßler) was used in the current study, and was applied as an inoculum consisting of the roots and growth substrate (1:1 sand:Agsorb® (a calcinated, attapulgite clay particle soil conditioner (Oil-Dri, Cambridgeshire, UK)) of *Plantago lanceolata* L. and *Trifolium pratense* L. plants colonised with *F. mosseae*, originally sourced from PlantWorks UK Ltd, Kent.

5.2.1.2 Experimental design

In our experiments, plants were colonised with AMF (+AMF) or non-colonised (-AMF) and infested with (+Aphid) or without pea aphids (-Aphid) in a factorial manner. The '+Aphid' treatment contained sub-treatments of aphids carrying no facultative symbionts (no H) or

one of three distinct strains of *H. defensa* ('H_{236}', H_{207}' and 'H_{216}') (Table 13), as detailed below. In all cases N = 8 with the exception of the '-AMF, +Aphid, -*H. defensa*' treatment where N = 7 due to failure of initial aphid survival.

Table 13. Semi-nested factorial design for AMF colonisation and aphid faculative symbiont status and strain.

AMF colonisation	Aphid secondary symbiont status
- AMF	- Aphid
+AMF	- Aphid
-AMF	+Aphid no facultative symbiont (no H)
+AMF	+Aphid no facultative symbiont (no H)
-AMF	+Aphid *H. defensa* strain 236 (H_{236})
+AMF	+Aphid *H. defensa* strain 236 (H_{236})
-AMF	+Aphid *H. defensa* strain 207 (H_{207})
+AMF	+Aphid *H. defensa* strain 207 (H_{207})
-AMF	+Aphid *H. defensa* strain 216 (H_{216})
+AMF	+Aphid *H. defensa* strain 216 (H_{216})

5.2.1.3 Plant growth

Broad beans (cultivar Sutton dwarf) were surface sterilised with 4 % (w/v) sodium hyperchlorite and germinated in a mixture of sand and Agsorb® (Oil-Dri, Cambridgeshire, UK) at a ratio 3:5 (v:v). Two weeks later plants were removed from the germination substrate and repotted into 0.5 L of sand 1:1 Agsorb ® (v:v) mixed with 0.25 g L^{-1} slow release organic fertiliser (sterilised bonemeal (Vitax, Leicestershire, UK)) and 25 g mycorrhizal inoculum to generate '+AMF' treatments. The Agsorb® was washed 1:2 (v:v) with dH_2O to remove excess solutes. Non-AMF (-AMF) treatments were generated in the same way although the mycorrhizal inoculum was autoclaved twice before addition and a non-fungal microbial filtrate was added as described by Hodge (2001). Plants were grown in a Snijder cabinet at 20°C with 150 µmol m^{-2} s^{-1} PAR provided by florescent tubes (a mixture of two brands; Osram GmbH, Munich, Germany and Phillips UK Ltd, Surrey, UK) a 16 h photoperiod and 65 % humidity when the lights were on, and 60 % humidity when the lights were off. Plants were watered with dH_2O as needed and fed weekly with 50 mL low P nutrient solution as Leigh, Hodge and Fitter (2009).

5.2.1.4 Aphid treatments

Aphid cultures were reared on broad bean at 20°C. Aphid culture plants were grown in F2 +S Levington compost. Adult aphids were removed from cultures and allowed to larviposit for 48 hours on broad bean leaves suspended in 2 % agar in Petri dishes (9 cm diameter). The mothers were then removed and the offspring were allowed to remain on leaves in the Petri dish for five more days. Four weeks post transfer of seedlings to AMF treatment pots and a total of six weeks after planting three 6 ± 1 day old pea aphids were added to

the bases of the stem of each aphid treatment experimental plant. No aphids were added to '-Aphid' treatments and all plants were covered in 70 cm x 15 cm polymer bread bags to prevent aphid escape and transfer between plants.

5.2.1.5 Harvest

A period of 17 days after aphid addition aphids were removed from the plants and stored at -20°C overnight before lyophilising using a Lyotrap (LTE Scientific Ltd, Oldham, UK) and weighing. To investigate possible top down effects of aphid feeding on AMF proliferation below ground, cores, 1 cm in diameter, of the plant growth substrate were removed and stored at 4°C overnight before hyphal extraction as in Staddon, Fitter and Graves (1999) and hyphal length density (HLD) of the extraradical mycelium was calculated using the gridline intercept method (Hodge 2003). Sub fractions of roots were stored in dH_2O at 4°C overnight before staining for intraradical root length colonisation (RLC) assessment using the acetic acid ink method (Vierheilig *et al.* 1998) modified for a 2 h clearing step in 10 % KOH at 70°C and stained using 5 % Pelikan® brilliant black ink and 5 % acetic acid in dH_2O. The remaining plant material was divided into root, leaf, and stem fractions with any flowers included as non-photosynthetic material with the stem and dried at 70°C for 72 h.

Plant material was milled using a MM440 (Retsch, GmbH, Haan, Germany) ball mill and pelleted before X-ray fluorescence for Si and P quantification using a Thermo Scientific™ portable X-ray fluorescence analyser (Reidinger, Ramsey & Hartley 2012). Milled plant material carbon (C) and N concentrations were analysed using an Elementar Vario El Cube (Elementar UK Ltd, Stockport, UK) C:N analyser.

5.2.2 Early plant defence signalling in AMF-aphid interactions

5.2.2.1 Experimental design and harvest

To eliminate the impacts of variation of aphid number and their location on the plant on defence gene expression, and to assess changes in gene expression early on in the interaction between AMF, aphids and the host plant, a second experiment was conducted. AMF colonisation and the presence of aphids were used as treatments in a fully factorial design. Plants were treated in the same manner as in the first experiment up until aphid addition and were set up in a randomised block design. Eight 4 ± 1 day old aphids per clip cage were added in two clip cages to the top two fully unfurled leaves of six week old plants. Empty clip cages were added to '-Aphid treatments'. Eight replicates of each treatment were set up with four replicates of each treatment harvested 6 hours post infestation (6 hpi) and the remaining four replicates of each treatment harvested 24 hours after aphid addition (24 hpi). Clip cages and aphids were removed from the plants and the leaf material inside the clip cage was immediately flash frozen in liquid nitrogen and stored

at -80°C until RNA extraction. Plants were assessed for AMF colonisation via acetic acid-ink staining as above. The data from one replicate of '+AMF, -Aphid, 24 hpi' was discarded as no AMF colonisation of the plant had occurred.

5.2.2.2 Gene expression analysis

RNA was extracted using a Qiagen (Hilden, Germany) RNeasy plant minikit with 30 µL RNase free H_2O. Subsequently, 1 µg of RNA was then DNase treated using Thermo Scientific™ (Waltham, Massachusetts, U.S.A) DNAse I, RNAse free before reverse transcription to cDNA using a Thermo Scientific™ RevertAid first strand cDNA synthesis kit according to the manufacturer's instructions using oligo DT primers. The reverse transcription protocol but without the addition of reverse transcriptase was also repeated for DNAse treated RNA samples to create reverse transcription controls to assess for DNA contamination. cDNA was diluted with 80 µL dH_2O prior to qPCR analysis.

Primer sequences for the RT-qPCR analysis of plant defence genes can be found in the Appendix (Table 18). Primers for VfLOX1 were designed using the primer BLAST software (Ye *et al.* 2012) using default parameters except the following: Primer length 18-23, Product length 150-200, Primer melting temp = 58 – 60, max difference = 2, No GC terminal forcing, GC content = 20 – 80 %, Max base repeat = 2. The VfLOX1 primer pair was tested for product specificity via sequencing the resulting PCR amplificon from cDNA sourced from pea aphid infested broad beans. Briefly, the PCR product was purified using a Qiagen QIAquick PCR purification kit, Sanger sequenced and then aligned to the VfLOX1 mRNA via BLAST. Gene expression analysis used the $^{\Delta\Delta}$CT method using the CYP2 gene as an endogenous control and qPCR was carried out in 20 µL reactions consisting of 2 µL cDNA, 10 µL FAST SYBR green ™ master mix, 0.7 µL each of 10 mM forward and reverse primers and 7.3 µL of dH_2O on a QuantStudio™ 3, 96 well, 0.1 mL block qPCR machine (Thermo Fisher Scientific™) with an initial 95°C denaturing step for 20 s, followed by 40 cycles of 95°C for 1 s, 60°C for 20 s, and a melt curve of 95°C for 1s, 60°C for 20 s then 95°C for 1 s.

5.2.2.3 Statistical analysis

Analysis was carried out using R (R Core Team 2016) version 3.3.2 (2016-10-31) and the packages 'lme4', 'lmerTest', 'lsmeans', 'multcomp' and 'multcompview'. As the experimental design was nested for the aphid performance assay, but not evenly due to the inevitable lack of '-Aphid, +*H. defensa'* treatments, data were analysed via a 'factor simplification' method of the final model: An intercept was set using the response variable and block as a random factor. The model was then subsequently updated with main (AMF presence, aphid presence, *H. defensa* presence and *H. defensa* strain) and then interaction effects (AMF*aphid presence, AMF**H. defensa* presence and AMF**H. defensa*

strain). For AMF and aphid traits, AMF presence and aphid containing interactions were respectively not included. The residuals of each step in the model were checked for normality using a Shapiro-Wilk's test and data was log, squared or square root transformed if the residuals were not normally distributed. An ANOVA was then used to compare each step of the update process. Where two factors interacted, a linear model using factors at the level of the interaction as explanatory variables and block as a random factor and a Tukey *post hoc* test was used in lieu of a *post hoc* test on the factor simplification model. For leaf Si and N concentration, data could not be transformed so that the assumption of normality of the residuals could be met, and as there is no non-parametric equivalent for the statistical model used, the model described was used regardless. As no interactions were significant for leaf Si or N concentration with the parametric test, it is unlikely that a more conservative non-parametric test would have reported significance.

For gene expression analysis from the clip cage experiment, the ΔCT values were analysed via a linear model with AMF colonisation and aphid presence as explanatory variables and block as a random factor. Residuals of the model were tested for normality with a Shapiro-Wilk's test.

5.3 Results

5.3.1 AMF-aphid-*H. defensa* interactions

5.3.1.1 AMF and aphid responses

Broad beans of '+AMF' treatments were well colonised averaging 52 % RLC ± 3.4 % S.E, 33 % arbuscules ± 2.8 % S.E, and 14 % vesicles ± 1.5 % S.E, whilst no fungal structures were detected in the roots of plants from the '–AMF' treatments. AMF colonisation significantly reduced the number of aphids on plants at harvest (-AMF: 130 ± 8 c.f +AMF: 103 ± 7; $X^2_1 = 7$, $P < 0.001$) whilst *H. defensa* presence or strain had no effect ($X^2_1 = <0.01$, $P = 0.952$). All statistical results are presented in Table 14. Aphids or their symbionts did not affect intraradical colonisation by AMF (RLC) ($X^2_1 = 0.1$, $P = 0.764$), the number of vesicles ($X^2_1 = 0.8$, $P = 0.364$) or arbuscules ($X^2_1 = 0.9$, $P = 0.352$), but there was a trend of aphid presence reducing the extraradical hyphae measured as HLD in AMF treatments (-Aphid: 1.17 ± 0.14 m hyphae g^{-1} dry substrate c.f +Aphid: 0.92 ± 0.06 m hyphae g^{-1} dry substrate; $X^2_1 = 3.7$, $P = 0.056$).

Table 14. Results of the model for the main factors of AMF colonisation, aphid presence and the semi-nested factors of secondary symbiont presence and strain and their interactions upon aphid characteristics, AMF characteristics and plant biomass and nutrient concentration responses. Degrees of freedom = 1, except for Symbiont strain and AMF*symbiont strain where degrees of freedom = 2.

Response variable	AMF		Aphid		Symbiont presence		Symbiont strain		AMF*Aphid		AMF*symbiont presence		AMF*symbiont strain	
	X^2	*P*	X^2	*P*	X^2	*P*	X^2	*P*	X^2	*P*	X^2	*P*	X^2	*P*
Aphid number	7.0	**0.008**			< 0.1	0.952	3.0	0.227			< 0.1	0.879	0.3	0.871
Root DW	7.1	**0.008**	0.3	0.555	0.7	0.416	5.2	0.073	5.2	**0.022**	0.1	0.701	1.0	0.596
Root [N]	13.5	**<0.001**	3.0	0.084	1.9	0.170	5.4	0.067	0.6	0.424	0.5	0.466	5.4	0.067
Root [P]	9.2	**0.002**	0.6	0.430	< 0.1	0.952	0.9	0.623	0.3	0.556	< 0.1	0.868	1.3	0.528
Root [Si]	< 0.1	0.974	1.1	0.294	1.7	0.193	4.0	0.137	3.7	0.055	0.3	0.566	3.5	0.171
Leaf DW	0.5	0.459	4.3	**0.037**	1.8	0.179	1.4	0.495	0.2	0.665	2.8	0.092	1.1	0.574
Leaf [N]	0.8	0.385	3.8	0.052	0.7	0.388	0.3	0.876	1.8	0.184	1.1	0.285	0.9	0.642
Leaf [P]	0.2	0.644	11.2	**0.001**	3.2	0.072	0.5	0.770	3.9	**0.049**	4.6	**0.031**	1.4	0.491
Leaf [Si]	< 0.1	0.875	0.2	0.679	0.9	0.355	0.1	0.916	0.3	0.605	< 0.1	0.884	0.1	0.958
Stem DW	0.6	0.447	0.7	0.395	0.04	0.845	0.5	0.797	1.0	0.317	1.1	0.292	1.0	0.605
Stem [P]	1.5	0.228	8.4	**0.004**	1.6	0.212	0.1	0.932	0.5	0.485	0.2	0.696	2.9	0.238
Stem [N]	0.4	0.526	7.5	**0.006**	0.6	0.435	1.6	0.447	0.2	0.697	0.8	0.378	3.9	0.143
Stem [Si]	0.6	0.456	12.6	**<0.001**	1.7	0.187	2.9	0.238	0.9	0.340	1.8	0.179	0.7	0.705
RLC			0.1	0.764	0.1	0.740	2.6	0.272						
Arbuscules			0.9	0.352	0.9	0.340	1.5	0.477						
Vesicles			0.8	0.364	0.5	0.499	0.2	0.923						
HLD			3.7	0.056	0.6	0.457	1.3	0.526						

[ ] =Concentration (mg g^{-1}), DW = dry weight (g), RLC, Arbuscules and Vesicles = % of root length, HLD = Hyphal length density (m hyphae g^{-1} dry growth substrate).

5.3.1.2 Plant biomass and nutritional responses

AMF colonisation and aphid presence impacted belowground plant biomass (measured as dry weight (g)), although below ground plant nutrient concentrations were only driven by AMF colonisation (Figure 14). AMF colonisation reduced root biomass in the presence, but not absence, of aphids (X^2_1 = 5.2, P > 0.05). Aphid presence had no effect on root nutrition while root N and P concentrations were increased by AMF colonisation (X^2_1 =9.2, P <0.005; X^2_1 = 13.5, P < 0.001, respectively). Neither AMF nor aphids affected root Si, although there was a trend for an interaction between the two (X^2_1 =3.7 P = 0.055).

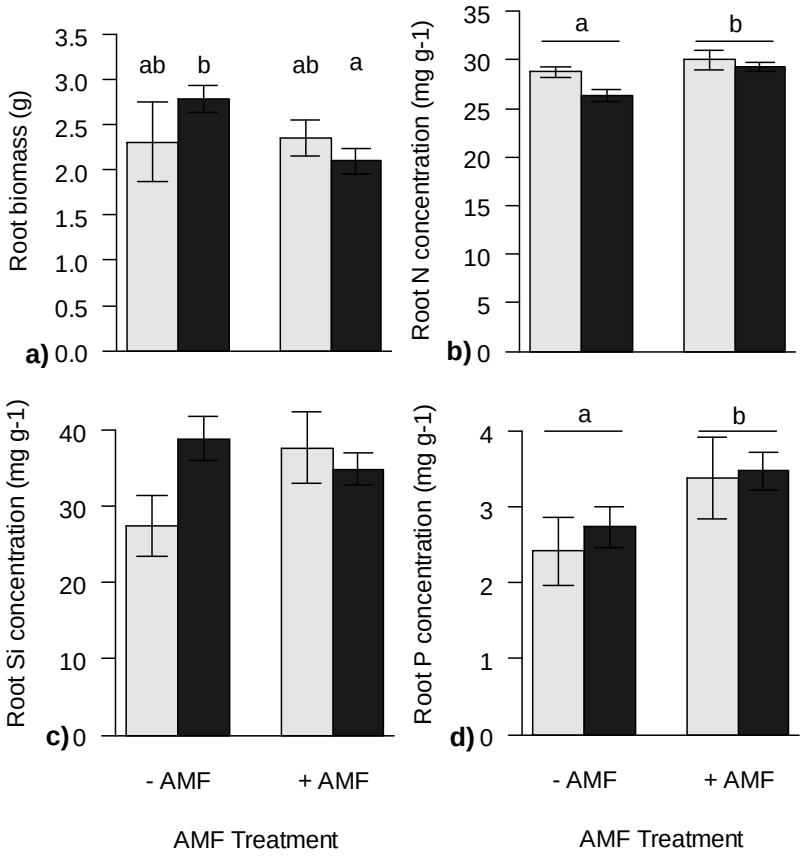

Figure 14. Mean root a) biomass (g) and concentrations (mg $g^{-1)}$ of (b) N c) Si (and d) P of broad beans colonised with or without AMF and hosting no aphids (light grey bars) or aphids (dark grey bars) at harvest. Different letters indicate significant differences at P = 0.05 based on a Tukey *post hoc* test. Error bars are ± standard error of the mean.

Whilst neither AMF nor aphids affected stem biomass, aphids reduced stem N, Si and P concentrations (X^2_1 = 7.5215, P < 0.01; X^2_1 = 9.2, P > 0.005; X^2_1 = 12.6, P <0.001 respectively; Figure 15).

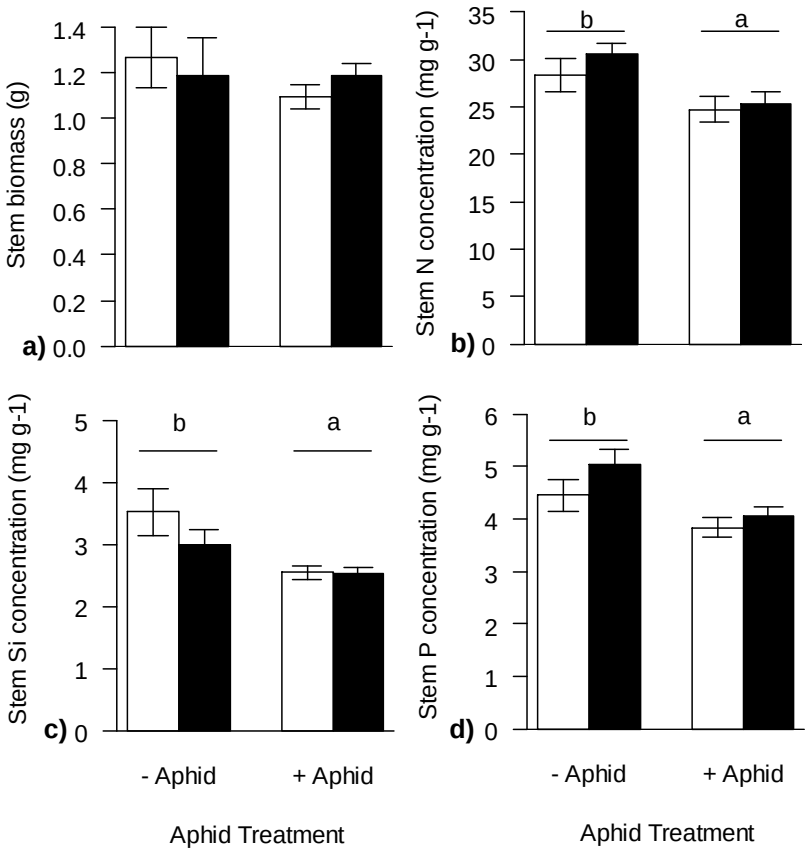

Figure 15. Mean stem a) biomass (g) and concentrations (mg $g^{-1)}$ of (b) N c) Si (and d) P of broad beans infested with or without aphids, and colonised with (black bars) or without AMF (white bars) at harvest. Different letters indicate significant differences at $P = 0.05$ based on a Tukey *post hoc* test. Error bars are ± standard error of the mean.

Moreover, leaf biomass was lower in plants hosting aphids (-Aphid: 1.22 ± 0.06 g c.f +Aphid: 1.06 ± 0.04 g; $X^2_1 = 4.3$, $P = 0.033$), and there was a trend of aphid feeding reducing leaf N concentration (50.86 ± 1.79 c.f 48.52 ± 0.43 mg g^{-1}; $X^2_1 = 3.8$, $P = 0.052$). Neither AMF nor aphids affected leaf Si concentration (3.59 ± 0.18 mg g^{-1}). Above ground, AMF only affected leaf P concentration and there was an interaction between AMF colonisation and aphid infection with *H. defensa* ($X^2_1 = 4.6$, $P = 0.031$); When colonised by AMF, aphids reduced leaf P concentration irrespective of the facultative symbiont treatment. However, in the absence of AMF colonisation, aphids carrying *H. defensa* reduced leaf P concentrations compared to aphids carrying no facultative symbiont (Figure 16).

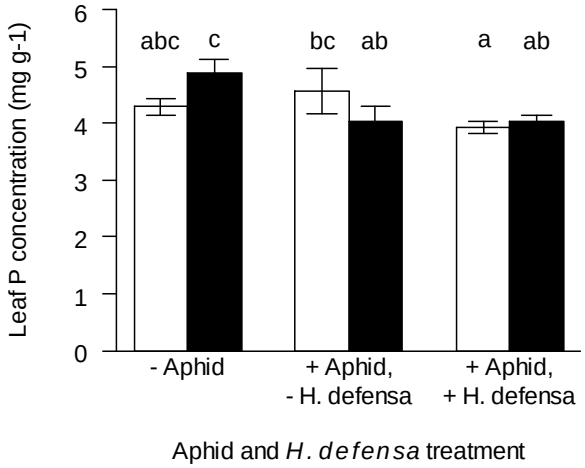

Figure 16. Mean leaf P concentration (mg g^{-1}) of broad beans colonised with (black bars) or without AMF (white bars) and subjected to or not to aphid feeding with aphids infected or not infected with *H. defensa*. Different letters indicate significant differences at $P = 0.05$ based on a Tukey *post hoc* test. Error bars are ± standard error of the mean.

5.3.2 Early plant defence signalling in AMF-aphid interactions

At 6 hours post infestation (6 hpi), aphid presence supressed the expression of the reactive oxygen scavenging gene SOD, suggesting the induction of a reactive oxygen species (ROS) burst typical of aphid feeding ($F_{1,9} = 8.7$, $P = 0.016$), whilst no such effect occurred at 24 hpi ($F_{1,11} = 0.3$, $P = 0.596$). AMF colonisation at either time point did not affect SOD expression (6 hpi: $F_{1,9} = 0.005$, $P = 0.970$, 24 hpi: $F_{1,11} = 1.9$, $P = 0.198$) (Figure 17a). At 24 hpi there was a trend of aphid presence supressing the JA pathway gene LOX1 expression ($F_{1,8.6} = 4.6$, $P = 0.062$). No such trend occurred earlier in the interaction at 6 hpi ($F_{1,9} = 0.4$, $P = 0.531$), and there was no effect of AMF on LOX1 gene expression at either timepoint (AMF, 6 hpi: $F_{1,9} = 2.3$, $P = 0.159$; AMF 24 hpi: $F_{1,8.6} = 0.2$, $P > 0.640$; Figure 17b).

At 6 hpi, plants colonised with AMF had significantly increased PR5 expression, a pathogenesis related protein involved in SA based defences ($F_{1,12} = 15.4$, $P = 0.002$), whilst aphid presence supressed PR5 expression ($F_{1,12} = 11.1$, $P = 0.006$). The interaction term between aphid presence and AMF was non-significant ($F_{1,12} = 0.2$, $P = 0.656$) (Figure 17c). At 24 hpi, the independent effects of AMF colonisation and aphid presence were no longer apparent on PR5 expression (AMF, 4 hpi: $F_{1,11} = 0.27$, $P = 0.615$; aphid 24 hpi: $F_{1,11} = 1.67$, $P = 0.222$). However, there was an interaction of AMF colonisation and aphid presence ($F_{1,11} = 7.3$, $P = 0.021$) with aphid induced upregulation, rather than downregulation of PR5 only occurring when the plant was colonised by AMF, although a post hoc test could not distinguish between treatments.

No significant effect of either treatment at either time point was detected for PR1 expression (AMF, 6 hpi: $F_{1,12} = 0.96$, $P = 0.346$; Aphid, 6hpi: $F_{1,12} = 0.42$, $P = 0.527$; AMF, 24 hpi: $F_{1,7.8} = 0.004$, $P = 0.950$; $F_{1,7.8} = 0.01$, Aphid, 24 hpi: $P = 0.924$ respectively; Figure 17d).

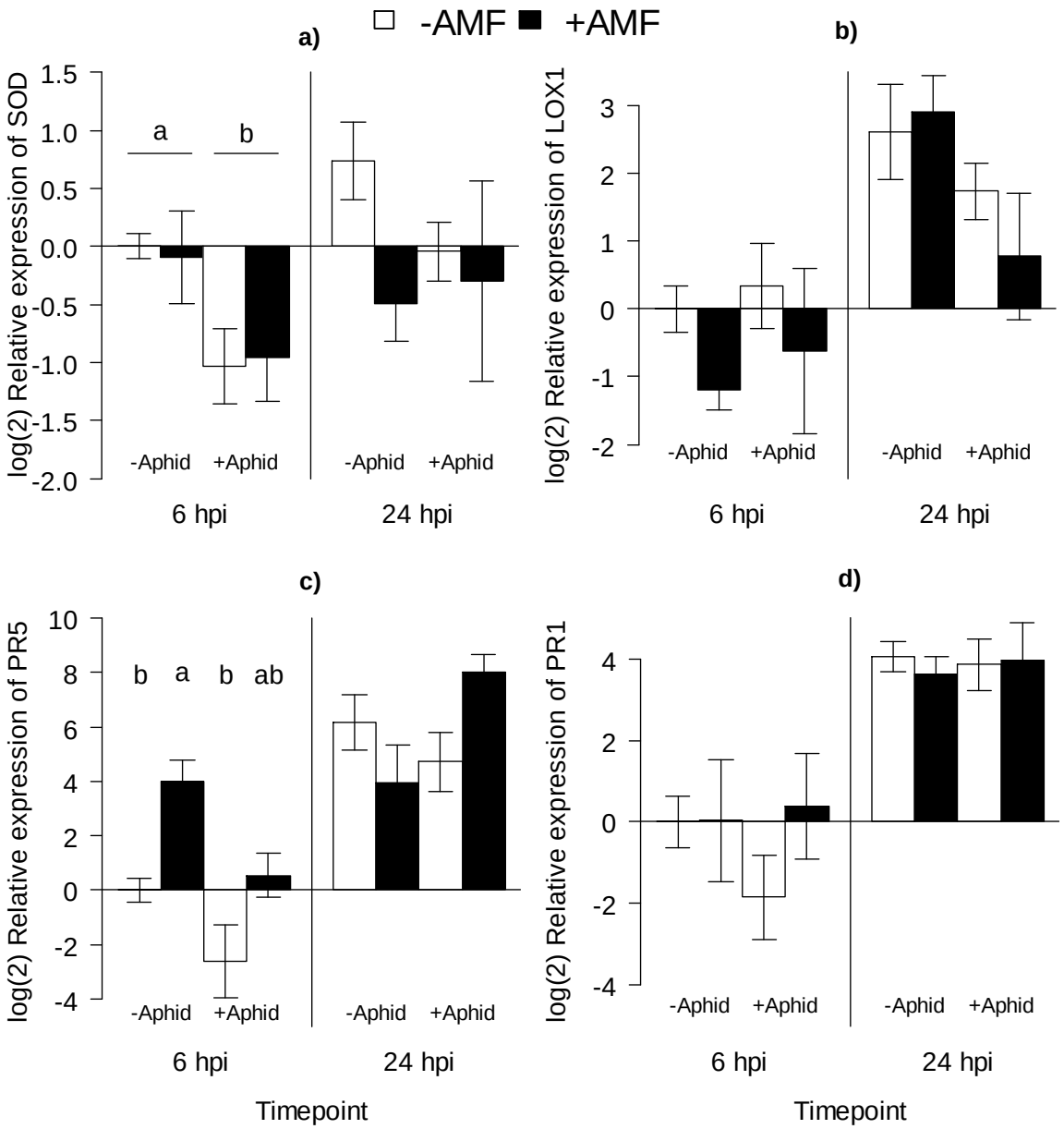

Figure 17. Expression levels of a) SOD b) LOX1 c) PR5 and d) PR1 in leaves of broad beans colonised with or without AMF and subjected to or not to aphid feeding sampled at 6 or 24 hours post infestation (hpi). Bars represent $\log_{(2)}$ transformed mean expression relative to non AMF, non-aphid control plants at 6 hpi calculated using the $^{\Delta\Delta}$CT method using the CYP2 gene as an endogenous control. Statistical analysis was carried out on ΔCT values within time points. Different letters over horizontal lines represent significant differences at $P = 0.05$ between the overall data the lines are over and different letters over individual bars represent significant differences between treatments, at $P = 0.05$ based on a Tukey *post hoc* test. Although a Tukey *post hoc* test could not identify significant differences between treatments at 24 hpi for c) PR5 expression, there was a significant interaction for AMF colonisation and aphid presence ($F_{1,11} = 7.3$, $P < 0.05$). Error bars are ± standard error of the mean (n = 4 except for + AMF, - Aphid at 24 hpi where n = 3).

5.4 Discussion

This study investigated the underlying plant nutrition and defence signalling related mechanisms in AMF-aphid interactions, and whether the aphid facultative symbiont *H. defensa* modulates the response of aphids to these changes. As predicted by our first hypothesis, aphid performance was affected by AMF colonisation, and aphid populations were lower on AMF plants. Regarding the second part of this hypothesis, AMF colonisation had little effect on above ground plant biomass and tissue nutrient status. However, there was an interaction between AMF and aphids upon plant defence signalling. This is the first time such an interaction has been associated with altered aphid performance and suggests that plant defence priming was a driver of the MIR against aphids reported. Contrary to our second hypothesis, the induced resistance against aphids occurred regardless of *H. defensa* presence or strain, although *H. defensa* did modulate the interaction between AMF and aphids upon leaf nutrition. Finally, although aphid feeding did not affect the intraradical colonisation of plants by AMF, there was a trend of aphid herbivory negatively affecting AMF structures outside of the root, i.e. the external hyphae.

5.4.1 Tri-trophic interactions between AMF-aphids- and an aphid associated facultative symbiont on plant physiology and nutrition

We hypothesised that AMF could alter aphid performance via the nutritional status of the plant. As AMF colonisation had little effect on above ground plant nutrition, it is unlikely that plant nutrition drove the reduced aphid performance in the current study. Aside from AMF interacting with aphid and *H. defensa* presence upon leaf P, aphids mostly impacted above ground plant biomass and N and P status (negatively). This is similar to previous reports of *A. pisum*-*V. faba*-AMF interactions (Babikova *et al.* 2014a). Moreover, *A. pisum* reduced stem Si concentration in the current study, whilst AMF colonisation had no effect. AMF colonisation can improve plant Si uptake, which may lead to resistance to root herbivores (Frew *et al.* 2017b) but this was not replicated in the above ground interaction investigated here. Si may be actively taken up by plants or passively acquired by transpiration (McLarnon *et al.* 2017). As Si uptake in *V. faba* is proposed to be a mostly passive process in the absence of a damage response (Liang, Si & Romheld 2005), reduced transpiration due to aphid feeding reducing leaf biomass in the current study may have led to lower levels of stem Si.

The multitrophic effects on plant leaf P are more complex; leaf P concentrations were reduced by aphids both with and without *H. defensa* when the plants were colonised by AMF, whilst only feeding by aphids carrying *H. defensa* reduced leaf P concentrations in non-AMF plants. AMF may suppress the role of P acquisition by their host plant (Smith, Smith & Jakobsen 2003), and thus if aphids negatively affect AMF this could lead to

detrimental effects on plant P uptake. We hypothesised that aphids would reduce both intraradical and extraradical AMF structures. Contrasting with a previous report (Babikova *et al.* 2014a), aphids did not affect AMF RLC, perhaps due to a shorter length of feeding in the current study (17 days c.f 28 days). However, we report for the first time, the effect of aphids on AMF extraradical hyphae (HLD) in which a strong negative trend occurred, potentially explaining the reduced leaf P status of plants hosting both aphids and AMF. Root biomass was lowest on plants hosting both AMF and aphids, suggesting that above ground herbivory may have limited the fixed C available to the AMF provided by the plant host, as proposed by the C limitation hypothesis (Wallace 1987). Although *H. defensa* may supress plant defence signalling (Su *et al.* 2015; Frago *et al.* 2017), the reduction of *Solanum* spp. root biomass by *M. euphorbiae* carrying *H. defensa* suggests the facultative symbiont can also lead to the elicitation of a plant defence response (Hackett, Karley & Bennett 2013; Bennett *et al.* 2016). Evidence suggests that *H. defensa* may induce a plant defence response in the current system (see Methods for Chapter 5 Appendix; Results for Chapter 5 Appendix), although further study is required to investigate whether this is associated with changes to plant nutrition.

5.4.2 Plant defence signalling in early AMF-aphid interactions

We hypothesised that changes to aphid performance due to AMF colonisation may be linked to alterations in plant defence signalling pathways, and thus investigated JA, SA and ROS related gene expression early in the interaction. It has been proposed that plant defence signalling is more influential early in the plant-aphid interaction rather than later in the process (post 7 days of aphid infestation; (Maurya *et al.* 2018)). This hypothesis was supported by the patterns of plant defence gene signalling in the current study: there was no link between gene expression with aphid performance late in the interaction (Methods for Chapter 5 Appendix; Results for Chapter 5 Appendix). However, differences in the number and location of aphids in the later harvest of plant material for gene expression analysis in the current study could contribute to this variable response.

Common features in early plant-aphid interactions such as ROS induction (Lei & Zhu-Salzman 2015) and JA suppression (Schwartzberg & Tumlinson 2014), suggested by aphid suppression of SOD and LOX1 gene expression in the current study, were not modulated by AMF. This suggests that at the time points measured, JA signalling and ROS modulation were not drivers of the MIR reported. However, the expression of the SA pathway marker gene PR5 was constitutively upregulated by AMF and simultaneously supressed by aphids at 6 hours post infestation (6 hpi). Moreover, at 24 hpi there was an interaction between AMF colonisation and aphid performance on this gene. Interestingly, this increased SA signalling at 24 hpi did not coincide with a concomitant increased suppression of JA signalling as reported in previous *V. faba-A. pisum* studies

(Schwartzberg & Tumlinson 2014). SA and JA defence responses in the *V. faba-A. pisum* system are genotype dependent (Stewart *et al.* 2016), perhaps contributing to this variation between studies. There is no general trend of the effect of SA upon aphids (Zust & Agrawal 2016), and as *A. pisum* supress both JA and SA signalling on their preferred host plants (Sanchez-Arcos *et al.* 2016), this suggests that the augmentation of SA signalling by AMF in the current study may be involved in the MIR reported against aphids.

At 24 hpi, the constitutive upregulation of PR5 by AMF was masked by an overall increase in PR5 expression, even in non-AMF plants. Whilst it is difficult to disentangle this from the effect of the clip cage on the leaf, difference in gene expression between the two timepoints may be in part due to the diurnal regulation of PR5 (Bhardwaj *et al.* 2011). Diurnal regulation of plant defences coincide with the peak activities of pathogens or pests at different times of day, reducing plant resource use (Lu, McClung & Zhang 2017), and *A. pisum* are 7 times more active during the day than at night (Joschinski *et al.* 2016) coinciding with the 24 hpi time point in the current study.

The small, but, significant effects of AMF colonisation on root biomass and plant nutrition, as well as PR5 induction in the absence of aphids in the current study excludes the MIR reported here from being soley attributed to plant defence priming (Martinez-Medina *et al.* 2016). However, the increased responsiveness of AMF colonised plants to aphid induced PR5 suppression reinforces that priming is an intrinsic part of induced resistance (Mauch-Mani *et al.* 2017). Unlike in MIR against leaf chewing herbivores (Song *et al.* 2013), the expression of JA genes was not modulated by AMF in the current study. JA defences are commonly associated with leaf chewing herbivores and biotrophic pathogens, whilst SA defences are associated with biotrophic pathogens and phloem feeding herbivores such as aphids (Berens *et al.* 2017). AMF have been shown to prime SA (as well as JA) inducible PR gene expression in potato against (hemi)biotrophic fungal pathogen which have biotrophic and a necrotrophic stages, such as against *Phytophthora infestans* (Mont.) de Bary (Gallou *et al.* 2011) and *Magnaporthe oryzae* (Campos-Soriano, Garcia-Martinez & San Segundo 2012). It is possible that as aphid feeding keeps the plant tissue they feed upon alive, MIR against aphids may reflect that against (hemi)biotrophic pathogens, rather than that against leaf chewing herbivores.

5.5 Conclusions

Understanding multitrophic interactions is important in predicting the use of biological controls in agricultural systems. Moreover, as all the organisms involved could contribute to plant nutrition and defence outcomes, each player can influence plant productivity. Here we demonstrate that AMF may modulate plant defence signalling pathways to confer MIR

to the pea aphid (*A. pisum*). The aphids' facultative symbiont *H. defensa* may not protect the host from these effects, although it may alter how the aphid affects plant nutrition. Early MIR responses to *A. pisum* differ to that of leaf chewing herbivores, potentially due to the unique feeding behaviour of aphids.

6 General Discussion

6.1 Summary of project aims

Global food security faces the dual challenges of increasing food production alongside improving agricultural sustainability (FAO 2011; DEFRA 2018). Currently, conventionally managed crop systems often employ prophylactic applications of synthetic pesticides, which could introduce unnecessary risks of pesticide resistance and non-target effects (Ramsden *et al.* 2017). Harnessing the benefits of multitrophic interactions between crops, their beneficial soil microbes and plant pests could reduce reliance on chemical pesticides (Pineda, Kaplan & Bezemer 2017; Thirkell *et al.* 2017). For example, arbuscular mycorrhizal fungi (AMF) can confer increased plant resistance to a variety of pests (Koricheva, Gange & Jones 2009; Yang *et al.* 2014). However, the impact of AMF colonisation on aphid performance via the shared plant host is variable (Gange & West 1994; Guerrieri *et al.* 2004; Simon *et al.* 2017; Tomczak & Muller 2018), which is one reason that currently limits the use of this multitrophic interaction for sustainable agriculture. Understanding the physiological and chemical mechanisms that drive the effects of AMF upon aphids will contribute to predicting the factors that lead to this variation. Therefore, several knowledge gaps in the understanding of this multitrophic interaction were identified in Chapter 1, including; understanding the role of plant nitrogen (N) nutrition and defence signalling in AMF-aphid interactions, the impacts of aphid herbivory on the plant host on AMF, and the role of aphid associated facultative symbionts (FS) in AMF-aphid interactions. The aims of this project were based around these (Figure 18) and were specifically:

1. To investigate the impact of AMF delivered N upon plant performance, alongside the performance of the plant's associated aphid herbivore.
2. To investigate the interplay between jasmonic acid (JA) and salicylic acid (SA) pathway defence signalling in AMF-aphid interactions.
3. To investigate the impact of aphid infestation of the shared plant host upon extraradical AMF structures and AMF communities.
4. To assess whether or not aphid infection by FS and FS strain type influences the response of aphids to AMF colonisation of the shared plant host.

This project used two plant-aphid species model systems in glasshouse experiments to investigate the impact of AMF upon the performance of aphids sharing the host plant (Chapter 2, Chapter 3, Chapter 5). A stable isotope experiment was used to elucidate the effects AMF acquisition of N on the plant host and its associated aphid pests (Chapter 3). Molecular methods were employed to investigate the modulation of key defence signalling

pathways during the early stages of mycorrhizal-induced resistance to aphids (Chapter 5), and how aphid facultative symbionts (FS) modify the effect of AMF upon aphid performance was also investigated (Chapter 5). The impact of aphid infestation on AMF colonisation of the plant root and external hyphae was also investigated (Chapter 4, Chapter 5) and via next generation sequencing, the effect of aphid herbivory of the shared plant host on AMF communities in a conventional agricultural system was examined (Chapter 4).

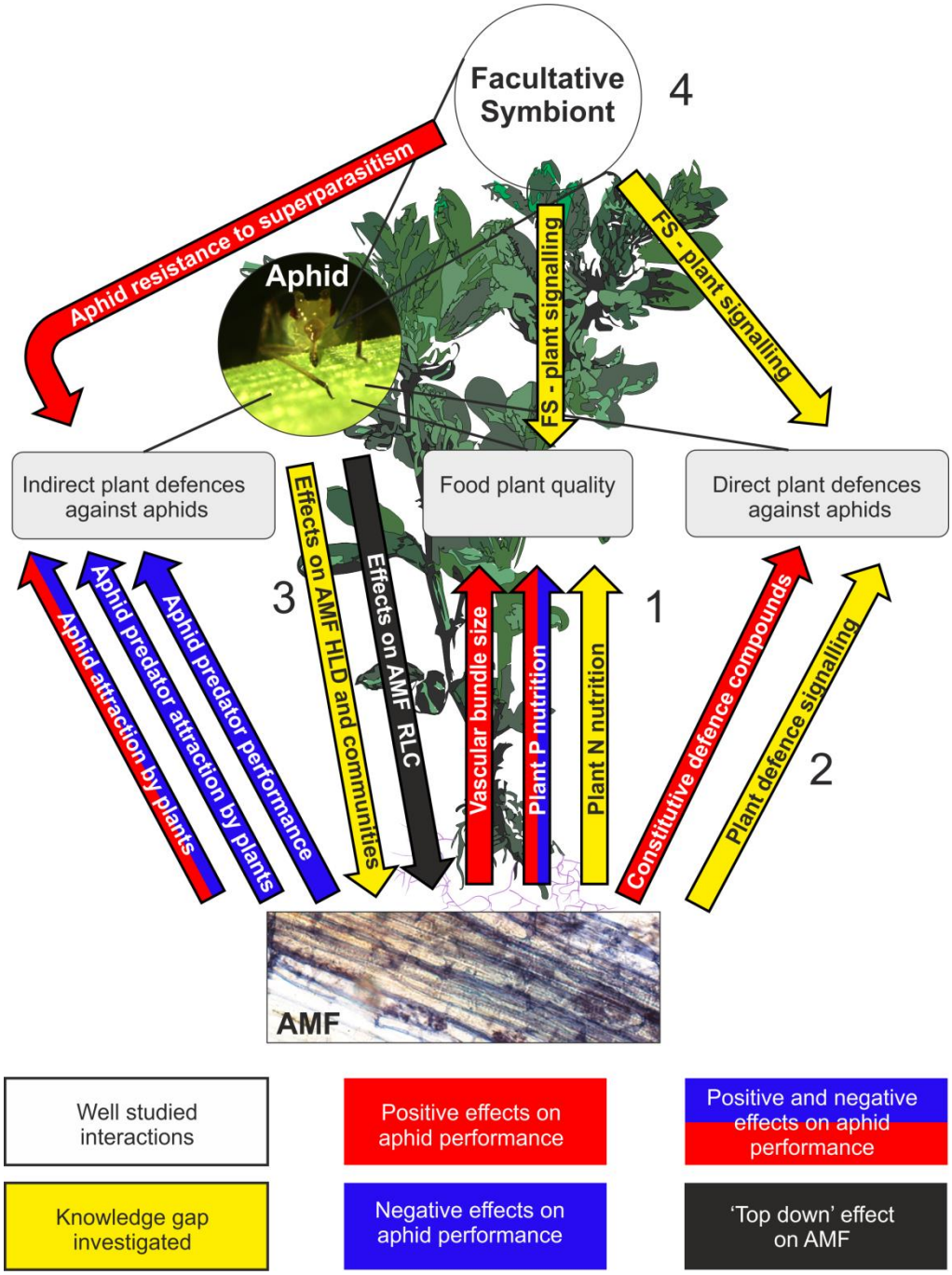

Figure 18. Representation of potential interactions between arbuscular mycorrhizal fungi (AMF) and aphids. Although many such interactions could occur, only well studied interactions (clear testing of factor in the literature; red/black/blue) and Key Knowledge Gaps investigated in the current project (yellow) are shown. Key Knowledge Gaps include 1) The effect of AMF access to nitrogen sources upon aphid and host plant performance and nitrogen status. 2) Multitrophic effects on plant defence signalling and plant defence priming early in the AMF-aphid interaction. 3) The 'top down' effects of aphids on AMF hyphal length density (HLD) and community structure. 4) The impacts of aphid facultative symbionts (FS) upon aphid responses to AMF mediated changes to plant quality and defence. RLC refers to root length colonisation. Image of plant modified from Clipart courtesy FCIT (http://etc.usf.edu/clipart/).

6.2 Implications of results, and suggestions for further study

6.2.1 Alterations to plant nutrition and defence as driving mechanisms of the effects of AMF on aphid performance (Knowledge Gap 1 and 2)

Often, the impacts of AMF colonisation of a shared host plant upon aphid performance are ascribed to potential alterations to the plants nutritional quality as a food source, or to the modulation of plant defence (Gange & West 1994; Wurst *et al.* 2004; Meir & Hunter 2018b). However, as AMF often alter both of these simultaneously, it can be difficult to disentangle the two (Mauch-Mani *et al.* 2017). Understanding which is important in the outcome of the AMF-aphid interactions could aid in using AMF to supress aphids in agricultural settings. N is a limiting nutrient in the diet of aphids (Douglas 2006; Butler, Garratt & Leather 2012), and therefore alterations to plant N uptake is a likely mechanism for AMF altering the food quality of plants for aphids. AMF can also enhance phosphorus (P) and silicon (Si) uptake (Harrison, Dewbre & Liu 2002; Garg & Bhandari 2016; Garg & Singh 2018), whilst with chemical fertilisation these nutrients can negatively affect aphid performance on host plants (Costa, Moraes & DaCosta 2011; Dias *et al.* 2014). However, the complexity of these interactions is highlighted by the findings in this thesis: aphids did not respond to AMF induced changes to plant N (Chapter 2, Chapter 3), whilst aphid performance was impacted by AMF colonisation when plant N was not altered (Chapter 5). This is similar to the contrasting effects of AMF- induced changes to plant N observed in the literature (Hempel *et al.* 2009; Babikova *et al.* 2014a; Williams, Birkhofer & Hedlund 2014; Tomczak & Muller 2017) and suggests that factors other than N are important in AMF-aphid interactions. By labelling the N sources that AMF can access and deliver to the plant, whilst simultaneously measuring aphid and plant N status (Chapter 3), it was revealed that via the plant, AMF can deliver N from below ground nutrient sources to aphids, but the effects of AMF colonisation upon plant and aphid N *concentration* can be uncoupled. Although the exact mechanism of how this occurs requires further elucidation, this suggests that aphids can overcome changes in plant N quality due to AMF colonisation. Electrical penetration graphing (EPG) methods can be employed in AMF-aphid interactions (Garzo, Rizzo & Fereres 2018), and it was revealed that aphids may reduce plant host acceptance in the first eight hours of the interaction when N quality is lowered due to AMF. Combining EPG methods with long term studies similar to that presented in Chapter 3 could unpick whether aphids eventually increase their phloem feeding times to compensate for reduced plant N quality in AMF-aphid interactions.

A meta-analysis suggests no general trend of the effect of P fertilisation of the host plant upon aphid performance (Butler, Garratt & Leather 2012). However, plant P status can indirectly impact aphid performance in some cases, for example by altering N availability (Tao & Hunter 2012). Similar to the impact of AMF-induced changes to plant N status,

throughout this project whenever AMF impacted plant tissue P status, aphid performance was not affected (Chapter 2 and Chapter 4), and AMF changes to aphid performance occurred when AMF colonisation did not alter the P status of plants hosting aphids (Chapter 5). Moreover, in both broad bean (*Vicia faba* L.) and barley (*Hordeum vulgare* L.), AMF colonisation did not increase plant Si uptake in response to aphid feeding (Chapter 2, Chapter 5). This contrasts with the role that Si uptake can play in below ground AMF-induced resistance, where increased Si uptake in AMF colonised roots is associated with protection against root chewing herbivores (Frew *et al.* 2017a). Taken together, plant tissue concentrations of N, P or Si measured throughout this project were not likely driving mechanisms of the outcomes of the AMF-aphid interactions studied.

Conversely, Chapter 5 revealed that reduced aphid performance can be associated with AMF-induced plant defence signalling modulation. As well as inducing constitutive defence responses, AMF colonisation can lead to 'priming' responses, where an augmented plant defence response occurs only in response to pest attack (Cameron *et al.* 2013; Martinez-Medina *et al.* 2016). AMF colonisation constitutively upregulated the SA pathway associated gene PR5 in the absence of aphid feeding, but this gene was also induced in response to aphid feeding later in the interaction (Chapter 5). This suggests that both constitutive changes to, and priming of, plant defence signalling contributed to the increased resistance to aphids conferred by AMF colonisation (Chapter 5). Moreover, Chapter 5 reinforces that AMF-induced plant defences can occur independently of large changes to plant nutrition (Fritz *et al.* 2006; Liu *et al.* 2007). As plant defence signalling responses may depend on genetic and developmental contexts (Stewart *et al.* 2016; Zhang *et al.* 2018), further study should investigate whether the augmentation of SA pathways by AMF colonised plants is a general trend across AMF-aphid interactions.

Nevertheless, this is the first documentation of plant defence signalling pathways being modulated in AMF-aphid interactions (Maurya *et al.* 2018). As such, this project contributes to the understanding of why variation in aphid performance could occur. Priming in AMF-plant-pest interactions is proposed to occur via the activation and suppression of plant defence genes during root colonisation by AMF (Cameron *et al.* 2013; Perez-de-Luque *et al.* 2017). The level of AMF colonisation of the plant can determine AMF impacts on aphids (Garzo, Rizzo & Fereres 2018; Maurya *et al.* 2018; Meir & Hunter 2018a). Plants likely supress high levels of AMF colonisation via defence signalling pathways (Blilou, Ocampo & Garcia-Garrido 1999; Lopez-Raez *et al.* 2010). Thus, it is proposed (Meir & Hunter 2018a) that varying levels of AMF colonisation could lead to different levels of defence gene modulation, impacting the plants response to aphids. This theory is given weight by the role of plant defence signalling in AMF-aphid interactions (Chapter 5). Furthermore, as AMF-induced priming can be augmented by

beneficial soil bacteria (Perez-de-Luque *et al.* 2017), the identity of soil microbes other than AMF is also important to consider.

As aphid generation times are short, and are impacted by AMF treatments (Simon *et al.* 2017), collecting plant material unconfounded by aphid number whilst avoiding mechanical manipulation of the host plant remains a challenge at late stages of the AMF-aphid interaction. This may contribute to the difficulties in associating AMF-aphid interactions with plant defence gene expression at timepoints later than the first few days (Chapter 5; Maurya *et al.* 2018). However, the regulation of plant defence pathways can differ throughout the course of plant-pest interactions (Pineda *et al.* 2012; Stewart *et al.* 2016). Moreover, although JA and SA signalling pathways are key in determining the defence response of plants, their effects may be fine-tuned by other plant hormones, including abscisic acid, gibberellic acid, and ethylene (Berens *et al.* 2017). Aphids (Mai *et al.* 2014; Stewart *et al.* 2016; Maurya *et al.* 2018) and AMF (Lopez-Raez *et al.* 2010; Fernandez *et al.* 2014; Maurya *et al.* 2018) can independently influence the expression of these hormones, and so their expression profiles throughout the course of the AMF-aphid interaction should be examined in the future. Using plant defence pathway knock out mutants can aid in disentangling the role of JA and SA in plant defence responses (Song *et al.* 2013; Zhang *et al.* 2018), and such methods could be employed in future AMF-aphid interaction studies.

Given the association of plant chemical defences (Meir & Hunter 2018b) and defence signalling (Chapter 5) in AMF-aphid interactions, plant defence modulation by AMF appears to be a driver of aphid performance. However, this does not exclude both plant defence and nutrition from impacting aphid performance, and multiple mechanisms could occur simultaneously within AMF-aphid interactions. Moreover, alterations to plant physiology (Simon *et al.* 2017) and as yet unknown mechanisms could also play roles. As amino acid concentrations and ratios in the phloem may be related to aphid performance (Ponder *et al.* 2000; Karley, Douglas & Parker 2002), the effect of AMF colonisation upon phloem amino acid concentrations merits further investigation. Further study should build hypotheses on how environmental, genetic and developmental factors may impact AMF-aphid interactions, but should not overlook the role of plant defence modulation highlighted here whilst still monitoring plant nutrition.

6.2.2 Effects of aphids upon AMF (Knowledge Gap 3)

If aphids are able to influence the way AMF colonise the shared host plant, this could in turn alter how the AMF affects the aphid. For example, the extraradical hyphae of AMF, and the community of AMF taxa colonising a host plant can influence plant host nutrition and defence responses (Leigh, Hodge & Fitter 2009; Babikova *et al.* 2013a; Malik, Dixon

& Bever 2016; Meir & Hunter 2018b). Thus, the third aim of this project was to investigate the impact of aphid infestation of the shared host plant on AMF extraradical hyphae and community structure, which was previously unstudied.

Aphids can increase, decrease, or have no effect on the intraradical colonisation levels of AMF (Babikova *et al.* 2014a; Vannette & Hunter 2014; Maurya *et al.* 2018; Meir & Hunter 2018a). Across all the experiments in this project, aphid herbivory of the host plant did not affect the proportion of plant roots colonised by AMF, but there was a trend of the number of AMF vesicles increasing in field-grown barley under aphid infestation (Chapter 4). Vesicles are AMF lipid storage organs, and can be indicators of more fixed C being transferred from the plant host to the AMF, and high levels can be associated with plant growth depression (Jin *et al.* 2017).

Differences in plant genotype and AMF species between Chapter 2 and Chapter 4 may explain why this trend was not shared between the two AMF-barley *S. avenae* studies. Levels of AMF colonisation can depend on plant genotype (Chapter 2; (Boyetchko & Tewari 1995; An *et al.* 2010; Ryan *et al.* 2016), and this suggests that different genotypes have differing levels of control over, or capacity to form the AMF symbiosis. *A. pisum* on broad bean tended to decrease AMF extraradical hyphal length density (Chapter 5) but *S. avenae* did not decrease the hyphal length density measured in a barley field (Chapter 4). It is likely that the soil extracted from the barley field system also contained non-AMF hyphae, which could have masked any impacts caused by the aphid. The difference in the response of AMF hyphae to aphid herbivory of the host plant could also be linked to aphids reducing the biomass of leaves in Chapter 5, but not Chapter 4. The carbon (C) limitation hypothesis (Wallace 1987; Wamberg, Christensen & Jakobsen 2003; Gange 2007) proposes that alterations to the availability of the host plant imposed by herbivory could influence extraradical mycorrhizal structures. However, AMF proliferation into nutrient patches is not reduced by shading of the host plant (Hodge & Fitter 2010), and above ground herbivore induced plant defence signalling responses can also be carried below ground (Park & Ryu 2014; Maurya *et al.* 2018). Thus, the limitation of nutrients other than C, or the activation of plant defence signalling could also be important in aphid suppression of AMF. Similar to the above ground investigation of plant-mediated effects of AMF colonisation upon aphids, the elucidation of the effects of aphids upon AMF would benefit from simultaneous analysis of plant nutrition and defence. The abundance of certain AMF families also tends to be increased when plants host aphids (Chapter 4). Whether these tendencies for aphids to impact AMF community or external hyphal production leads to an alteration in AMF function should be explored.

6.2.3 Effects of secondary symbionts in AMF-aphid direct interactions (Knowledge Gap 4)

Many aphids carry bacterial facultative symbionts (FS) in natural systems (Zytynska & Weisser 2016). FS offer the aphid host a variety of benefits including the ability to colonise new hosts (Tsuchida, Koga & Fukatsu 2004; Tsuchida *et al.* 2011) and protection against abiotic stresses and biotic antagonists (Montllor, Maxmen & Purcell 2002; Oliver *et al.* 2003; Scarborough, Ferrari & Godfray 2005; Heyworth & Ferrari 2015). Only one previous study examined aphid FS and AMF simultaneously and this did not address whether aphid FS enhance or mitigate the effects of AMF colonisation of the host on aphid performance (Bennett *et al.* 2016). The fourth aim of this project was to investigate the impact of aphid FS, such as *Candidatus* Hamiltonella defensa Moran et al. (henceforth referred to as *H. defensa*), upon AMF induced changes to aphid performance (Chapter 5). It was hypothesised that *H. defensa* would aid the aphid in overcoming plant resistance induced by AMF colonisation. Aphid performance did not depend on *H. defensa* presence in this experiment, suggesting that the FS did not mitigate the increased resistance given to the plant by AMF. Furthermore, there is evidence that the plant defence response to aphids was increased when aphids were carrying *H. defensa* at late stages of aphid infestation (17 days). Proteins from the aphids' primary nutritional symbiont *Buchnera aphidicola* can elicit plant defences (Chaudhary *et al.* 2014). Whether, like the primary symbiont *B. aphidicola*, *H. defensa* produce compounds that elicit plant defences is currently unclear, but proteins from FS such as *Serratia symbiotica* can be found in the aphid honeydew excreted onto plants (Sabri *et al.* 2013). Comparative analysis of the salivary and honeydew proteomes of aphids not carrying FS with that of aphids carrying *H. defensa* could identify *H. defensa* derived proteins that could come into contact with the plant. As more information on the *H. defensa* genome has recently become available (Chevignon *et al.* 2018), these proteins could be synthesised in vitro and tested for the ability to elicit plant defence responses.

A variety of FS species and *H. defensa* strains can attenuate the attractiveness of *A. pisum* induced plant volatiles to aphid natural enemies (Frago *et al.* 2017). Conversely, the resistance of aphid hosts to aphid antagonists and heat stress conferred by FS can be very specific, and often depends on the species of aphid and/or FS (Zytynska & Weisser 2016; Guo *et al.* 2017), and even the FS strain (Oliver, Moran & Hunter 2005; Russell & Moran 2006). Thus, the impact of three different *H. defensa* strain types on AMF-aphid interactions were tested in Chapter 5. None of the strains used in this project protected the aphid from any adverse effects of AMF colonisation of the shared host plant. This suggests that AMF-induced changes in crop plants to aphid resistance may not be overcome by aphid FS, a promising result for the potential use of above-below ground

interactions in biocontrol and food security. However, whether this is a general pattern found across a variety of FS and aphid species, or is more specific merits further study.

6.2.4 Utilising AMF to control aphids in agricultural systems

A reduction in pesticide use could be achieved via following principles of integrated pest management (IPM), where a combination of factors, including crop genetics, agricultural management practices, enhancing natural pest antagonists and the harnessing of beneficial microbes aim to contribute to reduced pest severity. During IPM, pesticides are applied when pests reach a density able to induce yield damage high enough to justify chemical control (Barzman *et al.* 2015). Thus, by reducing the frequency of aphid populations reaching these densities, when AMF do increase aphid resistance by crops, this could contribute to reduced instances of pesticide use even in conventional systems.

This could have positive implications for food security by reducing the likelihood of pesticide resistance developing due to continued exposure of pests to chemical control (Gould, Brown & Kuzma 2018). A reduction in chemical control instances could also reduce non-target effects on pollinators and natural enemies to pests, providing further benefits through agricultural ecosystem services (van der Sluijs *et al.* 2015). Furthermore, mechanisms of AMF induced resistance such as defence priming have the potential to aid plants in resisting pests other than aphids (Mauch-Mani *et al.* 2017). However, manipulating the AMF colonisation of plants in an agricultural setting can be difficult: a microbe-free plant in a non-sterile setting is extremely unlikely (Partida-Martinez & Heil 2011), and so any commercial inoculums of AMF applied to soil will have to compete with the native soil communities in order to colonise plant roots in field agricultural systems. Thus, commercial inoculums may be of greatest impact when supplemented into glasshouse systems. In these systems soils are routinely sterilised or under strict control (Boyer *et al.* 2016; Valera *et al.* 2016). In arable systems, commercial AMF strains may establish in some cases (Schlaeppi *et al.* 2016; Imperiali *et al.* 2017; Symanczik *et al.* 2017), but others have low levels of persistence (Schlaeppi *et al.* 2016; Berruti, Lumini & Bianciotto 2017; Buysens *et al.* 2017; Imperiali *et al.* 2017; Lojan *et al.* 2017). In conventionally managed agricultural systems, where chemical and mechanical inputs result in unique lower diversity AMF communities tolerant to such practices (Jansa *et al.* 2002; Wetzel *et al.* 2014; Hartmann *et al.* 2015), new isolates of the AMF species already present in the field may persist better than species new to the system (Schlaeppi *et al.* 2016; Imperiali *et al.* 2017).

Research has begun to focus on the “steering” of soil microbial communities to supress above ground pests (Pineda, Kaplan & Bezemer 2017). One such method could be to inoculate a desired crop species grown in a sample of local field soil with pests in order to

impose a change in the soil microbial community associated with the plant. The resulting soil communities would then be selected depending on the suppression of the pest in a subsequent generation of plants (Pineda, Kaplan & Bezemer 2017). The results of Chapter 4 in the current project suggest this is plausible, as conventionally managed soil fungal communities and AMF taxa abundance tends to alter in response to aphid infestation of the plant host.

Continued research towards understanding the driving mechanisms contributing to the variation in the outcomes of AMF-aphid interactions could eventually inform this soil microbial community 'steering'. In order to obtain plant varieties associated with AMF communities that are resistant to aphids, physiological and chemical plant markers could be employed to screen during plant breeding and AMF community 'steering'. These markers could be chosen based on the mechanisms identified in this thesis and related studies (Simon *et al.* 2017; Meir & Hunter 2018b).

6.3 Conclusions

The effect of AMF upon the performance of aphids sharing the host plant can vary depending on many factors, limiting its use for agriculture. This project aimed to investigate what drives AMF aphid interactions, notably focusing on whether N acquisition by AMF, or modulation of plant defence signalling by AMF, is associated with effects on aphid performance. Moreover, interactions between AMF and aphids are bi-directional, and aphid infestation can impact AMF colonisation of the shared host plant. It is also possible that the multitrophic interaction could be altered by the presence of aphid FS. Thus, this project also aimed to investigate the impact of aphids on AMF, and *H. defensa* on AMF-aphid interactions. By conducting experiments across two model AMF-plant-aphid experimental systems, for the first time, it was shown that:

1) Whilst AMF can deliver N to aphids associated with the host plant, and increase the N concentration of the aphid's food source, this may not affect aphid performance. Moreover, the overall N uptake of aphids did not differ between AMF and non-AMF plants, which suggests that aphids may be able to alter their feeding behaviour, and/or metabolism to overcome differences in N quality of AMF and non-AMF plant hosts.

2) Reductions in aphid performance can be associated with plant defence signalling pathway modulation. It is likely that augmented SA signalling by AMF impacts aphid performance.

3) The carrying of *H. defensa* by aphids did not mitigate the impact of AMF colonisation of the host plant on aphid performance.

4) There is a tendency for aphid infestation of host plants to influence AMF structures outside of the plant root, such as extraradical hyphae. Aphid infestation also tends to alter the abundance of AMF families.

This research contributes to the identification of driving mechanisms in multitrophic interactions between AMF and aphids via the shared plant host. With further study, this can increase our understanding of what causes the variation reported in the outcome of AMF-aphid interactions. This knowledge could eventually inform the use of below- above-ground multitrophic interactions in sustainable agriculture.

Appendices

1. Appendix for Chapter 3

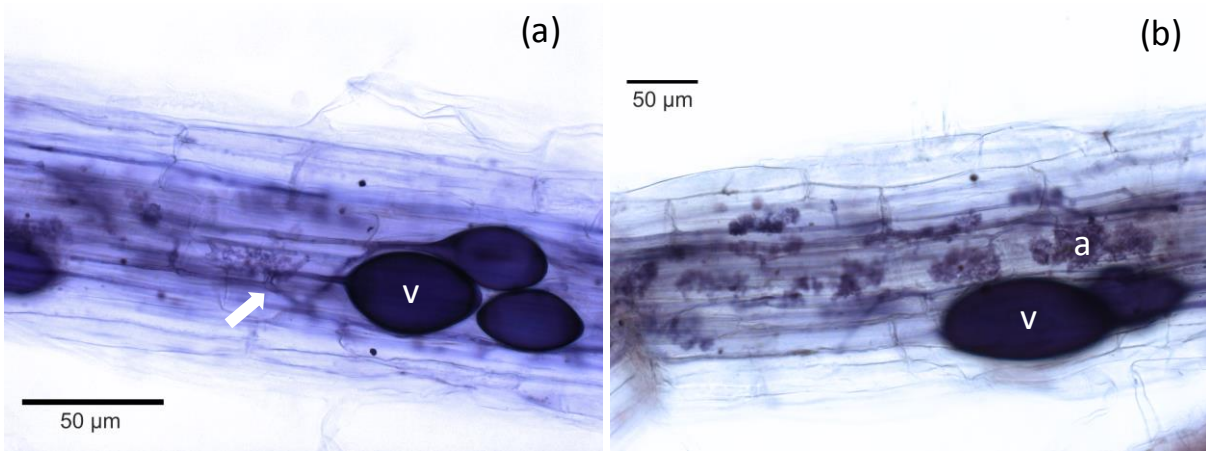

Figure 19. (a) and (b). Photomicrographs of the arbuscular mycorrhiza (AM) fungus *Funneliformis mosseae* in roots of barley (*Hordeum vulgare* L.) showing both arbuscule (a) and vesicle (v) structures. The arrow on Fig 19 a points to the trunk attachment of the arbuscule structure. Scale bars: 50 µm.

2. Appendix for Chapter 4

Table 15. Field site soil chemical analyses and agrochemical inputs used throughout the study

Soil analyses (sampled 12/05/17)		
P (Olsen's)	182 mg l^{-1}	
K (Ammonium nitrate extracted)	274 mg l^{-1}	
Mg (Ammonium nitrate extracted)	47 mg l^{-1}	
pH	7.4	
Organic matter % (Loss on ignition)	6.8	
Agrochemical inputs		**Date of Input**
Herbicides		
	Crystal 1.9 l^{-ha}	15/03/17
	Duplosan 1.74 l^{-ha} + Harmony 0.1 l^{-ha}	25/05/17
	Gal-Gone 0.5 l^{-ha}	03/06/17
	Axial 0.3 l^{-ha} + Agidor (Adj) 0.1 l^{-ha}	04/06/17
Fungicides		
	Siltra Xpro 0.4 l^{-ha}	25/5/17
	Chlorothalonil 1.0 l^{-ha} + Siltra Xpro 0.4 l^{-ha}	12/6/17
Plant Growth Regulators		
	Terpal 0.58 l^{-ha}	03/06/17
Fertiliser		
	YARA N35 + 7SO3 231 kg^{-ha}	16/03/17
	OMEX 0:10:15 623 kg^{-ha}	31/03/17
	YARA N35 + 7 SO3 280 kg^{-ha}	20/04/17

Table 16. Primer sets and PCR conditions used in nested PCRs for ‘Total fungi’ and ‘AMF specific’ amplicon sequencing.

Amplicon		Primer pairs	Cycling conditions	DNA used in reaction
Total fungi				
	Primary PCR	ITS1F (Gardes & Bruns 1993) to ITS4 (White *et al.* 1990)	5 mins @ 95°C; 35 cycles (30 s @ 94°C, 45 s @ 55°C, 90 s @ 72°C); 10 mins @ 72°C.	10 ng extracted DNA
	Secondary PCR (Illumina tagged primers)	GITS7 (Ihrmark *et al.* 2012) to ITS4 (White *et al.* 1990)	5 mins @ 95°C; 30 cycles (30 s @ 94°C, 45 @ 55°C, 90 s @ 72°C); 5 mins @ 72°C.	Total fungi primary PCR product (diluted 1:1000)
AMF specific				
	Primary PCR	AML1 to AML2 (Lee, Lee & Young 2008)	2 mins @ 95°C; 30 cycles (30 s @ 94°C, 30 s @ 59°C, 90 s @ 72°C), 10 mins @ 72°C.	10 ng extracted DNA
	Secondary PCR(Illumina tagged primers)	WANDA (Dumbrell *et al.* 2011) to AML2 (Lee, Lee & Young 2008)	5 mins @ 95°C; 30 cycles (30 s @94°C, 40 s @ 59°C, 90 s @ 72°C), 10 mins @ 72°C	AMF specific primary PCR product (undiluted)

Table 17. AMF VT (Virtual taxa) identified via AMF specific amplicon sequencing. Unassigned = could not be assigned to a singular VT.

Family	Species	VT
Acaulosporaceae	Acaulospora sp.	VTX00030
Ambisporaceae	Ambispora sp.	VTX00283
Archaeosporaceae	Archaeospora sp.	unassigned
Archaeosporaceae	Archaeospora sp.	VTX00245
Archaeosporaceae	Archaeospora sp.	VTX00338
Diversisporaceae	Diversispora sp.	unassigned
Diversisporaceae	Diversispora sp.	VTX00354
Gigasporaceae	Scutellospora sp.	VTX00052
Glomeraceae	Glomus sp	unassigned
Glomeraceae	Glomus sp.	VTX00064
Glomeraceae	Glomus sp.	VTX00065
Glomeraceae	Glomus sp.	VTX00105
Glomeraceae	Glomus sp.	VTX00143
Glomeraceae	Glomus sp.	VTX00199
Paraglomeraceae	Paraglomus sp.	VTX00281

3. Appendix for Chapter 5

Table 18. Primer pairs for genes of interest used in qPCR gene expression analysis.

Name	Gene name	Pathway	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')	Template accession #	Author of primer pair
VfPR1	Pathogenesis-related gene 1	SA	CAGTGGTGACATAACAGGAGCAG	CATCCAACCCGAACCGAAT	JQ043349.1	Cheng et al., (2012)
VfPR5	Pathogenesis-related gene 5	SA	TCTGTAACTCCACAAGGCGG	TGTATTATGACTTCCACGGCAA	JQ043350.1	Cheng et al., (2012)
VfSOD	Super-oxide dismutase	ROS scavenging	CTGCCGCCAAGAAAGCC	GGTCCTGTTGAGATACACCCATT	CAA39819.1	Cheng et al., (2012)
VfCYP2	Cyclophilin	Housekeeping	TGCCGATGTCACTCCCAGAA	CAGCGAACTTGGAACCGTAGA	AB012947	Gutierrrez et al., (2011)
Vf LOX1	Lipoxygenase 1	JA	CCTTCCACATCCTCAAGGAGA	CAACCAGTGGCTGACAAGTT	z73498.1	this study

3.1. Methods for Chapter 5 Appendix

At the harvest of the first experiment outlined in the main text (17 days post aphid addition), the topmost unfurled leaf was removed, frozen in liquid N and stored at -20°C. Four replicates from within each treatment were selected as those plants carried the number of aphids most similar to the number of aphids per plant averaged across all the '+Aphid' treatments. As aphid number did not differ between *H. defensa* strains, H_{207} was chosen at random to represent *H. defensa* infected aphids. RNA extraction and qPCR analysis occurred as outlined in the main text and statistical analysis was carried out using the 'factor simplification' method outlined in the main text although symbiont strain interactions were not analysed or included.

3.2. Results for Chapter 5 Appendix

There were no effects of the treatments on JA or SA marker gene expression in the leaf tissue collected at harvest in the first experiment (17 days post aphid addition), possibly due to large variability in aphid performance between treatments and in the location of aphids upon the plant (Table 19; Table 20). However, compared to aphid-free plants, the expression of the reactive oxygen species scavenging gene SOD was reduced in plants infested with aphids carrying *H. defensa* but not in plants fed on by aphids without this symbiont (Figure 20; Table 19).

Table 19. Results of the model for the main factors of AMF colonisation, aphid presence and the semi-nested factor of secondary symbiont (H_{207}) presence upon broad bean plant defence gene expression. Degrees of freedom = 1.

ΔCT	AMF		Aphid		Symbiont presence		AMF*Aphid		AMF* Symbiont presence	
	X^2	*P*	X^2	*P*	X^2	*P*	X^2	*P*	X^2	*P*
PR1	0.2	0.652	1.5	0.219	1.1	0.285	0.5	0.500	0.4	0.528
PR5	0.4	0.534	0.2	0.638	0.7	0.409	< 0.1	0.794	< 0.1	0.835
SOD	< 0.1	0.988	2.4	0.121	4.8	**0.029**	3.2	0.072	< 0.1	0.909
LOX1	0.3	0.558	< 0.1	0.969	< 0.1	0.772	< 0.1	0.772	1.1	0.270

Table 20. Mean relative expression levels (± 1 S.E) of JA and SA pathway dependent defence genes of broad beans colonised with or without AMF and subjected to or not to aphid feeding with aphids infected or not infected with *H. defensa* (H_{207}) at 17 days post aphid addition.

Relative expression	-AMF, -Aphid	+AMF, -Aphid	-AMF, no H	+AMF, no H	-AMF, H_{207}	+AMF, H_{207}
PR1	1.77 ± 0.89	1.65 ± 0.97	0.64 ± 0.16	1.05 ± 0.47	0.62 ± 0.34	0.97 ± 0.67
PR5	1.33 ± 0.46	1.00 ± 0.39	1.16 ± 0.35	0.80 ± 0.17	1.83 ± 1.01	2.10 ± 1.45
LOX1	1.32 ± 0.57	2.37 ± 1.6	0.92 ± 0.45	2.8 ± 1.62	1.60 ± 0.60	1.42 ± 0.58

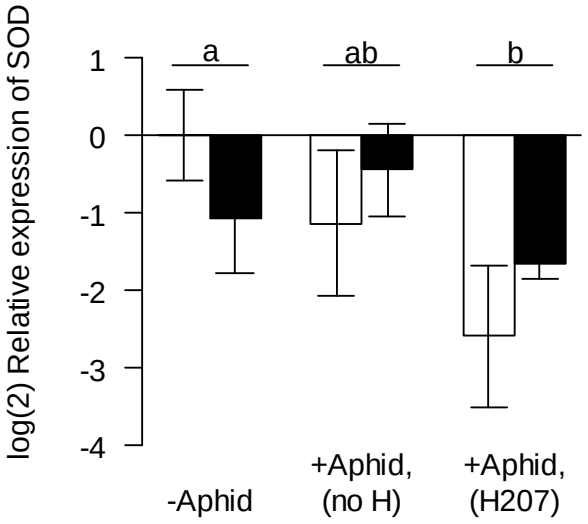

Figure 20. Expression levels of SOD in leaves of broad beans colonised with (black bars) or without AMF (white bars) and subjected to or not to aphid feeding with aphids infected or not infected with *H. defensa* (H_{207}) 17 days post aphid addition. Bars represent $\log_{(2)}$ transformed mean expression relative to non AMF, non aphid control plants calculated using the $^{\Delta\Delta}$CT method using the CYP2 gene as an endogenous control. Statistical analysis was carried out on ΔCT values. Different letters over horizontal lines represent significant differences at $P = 0.05$ between the overall data the lines are over based on a Tukey *post hoc* test. Error bars are ± standard error of the mean (n = 4).

Abbreviations

+AM:	Plant colonised by arbuscular mycorrhizal fungi
-AM:	Plant not colonised by arbuscular mycorrhizal fungi
AMF:	Arbuscular mycorrhizal fungi
ANOVA:	Analysis of variance
BLAST:	Basic local alignment search tool
C:	Carbon
cDNA:	Complimentary deoxyribonucleic acid
D.F:	Degrees of freedom
DAMP:	Damage associated molecular pattern
ΔCT:	Difference between cycle thresholds
DNA:	Deoxyribonucleic acid
DW:	Dry weight
EPG:	Electrical penetration graphing
ERM:	Extra-radical mycelium
FS:	Facultative symbiont
FW:	Fresh weight
H_0:	No facultative symbiont
H_{207}:	Hamiltonella defensa strain 207
H_{216}:	Hamiltonella defensa strain 216

H_{236}:	Hamiltonella defensa strain 236
HAMP:	Herbivore associated molecular pattern
HLD:	Hyphal length density
Hpi:	Hours post infestation
IRM:	Intra-radical mycelium
IRMS:	Isotope ratio mass spectrometry
JA:	Jasmonic acid
LOX:	Lipoxygenase
MAMP:	Microbial associated molecular pattern
MIR:	Mycorrhizal induced resistance
mRNA:	Messenger ribonucleic acid
N:	Nitrogen
N.E:	North east
N.W:	North west
NMDS:	Non-metric multidimensional scaling
OTU:	Operational taxonomic unit
P:	Phosphorus
PAR:	Photosynthetic active radiation
PCR:	Polymerase chain reaction

PERMANOVA:	Permutational multivariate analysis of variance
Pi:	Inorganic phosphorus form (orthophosphate)
PR:	Pathogenesis related (gene)
PRR:	Pattern recognition receptor
PTI:	Pattern triggered immunity
QIIME2:	Quantitative Insights Into Microbial Ecology 2
R gene:	Resistance gene
RLC:	Root length colonisation
RNA:	Ribonucleic acid
ROS:	Reactive oxygen species
RT-qPCR:	Reverse transcriptase - quantitative polymerase chain reaction
RWR:	Root weight ratio
S.E:	Standard error
SA:	Salicylic acid
Si:	Silicon
SOD:	Super oxide dismutase
VT:	Virtual taxa
XRF:	X-ray fluorescence

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